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New broad-spectrum antibiotics containing a pyrrolobenzodiazepine ring with activity against multidrug resistant Gram-negative bacteria.

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Abstract

It is urgent to find new antibiotic classes with activity against multidrug resistant (MDR) Gram-negative pathogens as the pipeline of antibiotics is essentially empty. Modified pyrrolobenzodiazepines (PBDs) with a C8-linked aliphatic-heterocycle, provide a new class of broad-spectrum antibacterial agents with activity against multidrug resistant Gram-negative bacteria, including WHO priority pathogens. The structure-activity-relationship established that the third ring was particularly important for Gram-negative activity. Minimum inhibitory concentrations for the lead compounds ranged from 0.125 – 2 mg/L for MDR Gram-negative, excluding *Pseudomonas aeruginosa,* and between 0.03 - 1 mg/L for MDR Gram-positive species. The lead compounds were rapidly bactericidal with >5-log reduction in viable count within 4 hours for *Acinetobacter baumannii* and *Klebsiella pneumoniae*. The lead compound inhibited DNA gyrase in gel-based assays, with an IC50 of 3.16 +/- 1.36 mg/L. This study provides a new chemical scaffold for developing novel broad-spectrum antibiotics which can help replenish the pipeline of antibiotics.

Introduction

The fight against bacterial infections is rapidly being lost as microbes develop multiple mechanisms to evade antibiotics. Antimicrobial resistance is one of the most significant health concerns worldwide. It is estimated that 25,000 people die each year in Europe and 23,000 in the United States because of resistant bacterial infections, with more than 2 million infections caused by drug resistant bacteria[1]. These values are expected to reach 10 million deaths globally by 2050, overtaking cancer and heart disease as the principal cause of death if no effective measures can be developed[2]. Of particular concern is the rapid emergence of multidrug resistant (MDR) Gram-negative pathogens, defined as having resistance to at least 3 frontline antibiotic classes, and increasing reports of essentially pan-drug resistant (PDR) isolates in the clinic. In the last 30 years, no major classes of broad spectrum antibiotics have been introduced to the market and recently approved agents like linezolid (2000), daptomycin (2003), and retapamutilin (2007) are active only against Gram-positive pathogens[3]. This makes it imperative that we discover new antibacterial drugs with a broad spectrum of activity or specificity for Gram-negative species, in order to help solve the crisis and provide new treatment options for MDR and PDR strains.

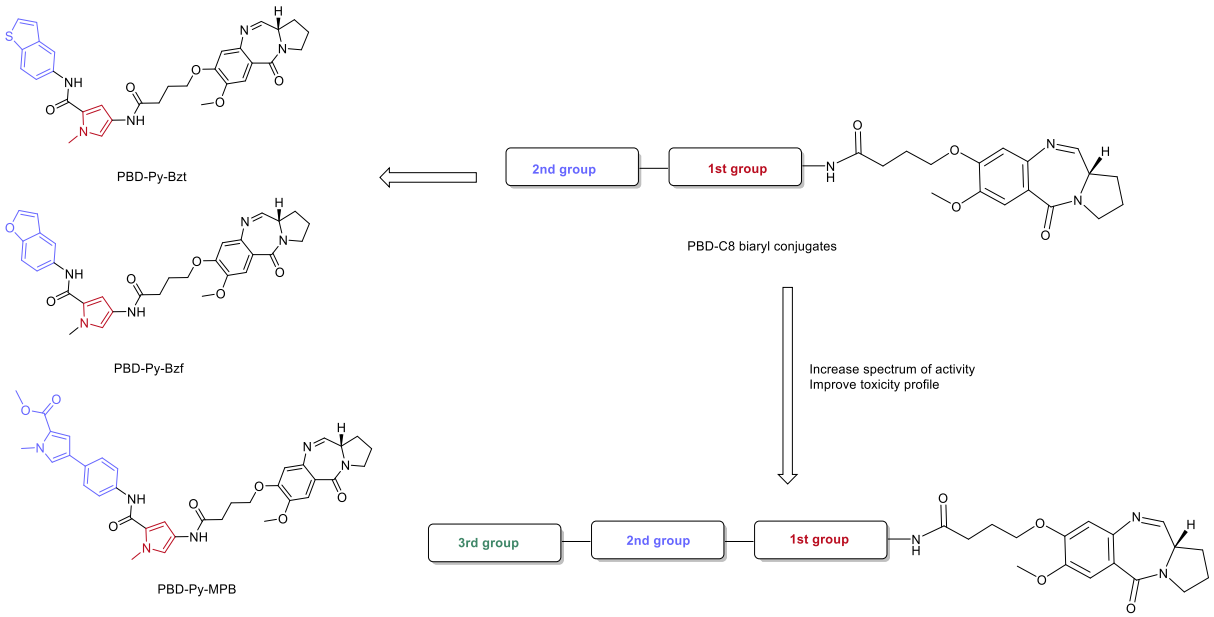


Figure 1: Reported antibacterial PBDs and structural modification employed to obtain compounds with activity against Gram-negative bacteria.

PBDs are naturally occurring molecules produced by *Streptomyces* bacteria whose family members include anthramycin and tomaymycin[4, 5]. PBDs have a soft N10-C11 imine electrophile that can

covalently bond to guanine bases[6]. They have been extensively studied as anticancer agents[7-11] and, more recently, a large number of PBDs are being clinically evaluated as payloads for antibody drug conjugates (ADCs) demonstrating the broad therapeutic utility of this chemical class[12, 13]. We recently described a series of C8-linked PBD monomers that showed activity against Gram-positive MDR strains[14, 15], which were able to inhibit *Staphylococcus* gyrase in a biochemical assay. It has been reported in the literature that the reason PBDs are only active against Gram-positive bacteria is due to their inability to cross Gram-negative membranes[16]. We designed and synthesized a new generation of C8-PBD monomers with an aliphatic third ring that showed, for the first time, notable activity against Gram-negative bacteria. Introduction of the aliphatic third ring improved the prokaryotic selectivity of the molecules and reduced eukaryotic toxicity of the molecules, as the third ring interfered with the DNA binding ability of these molecules.

The synthesized compounds showed broad spectrum activity against both MDR and PDR clinical ESKAPE strains with MICs ranging from 0.03 to 1 mg/L against Gram-positive species and 0.125 to 32 mg/L against Gram-negative species. The compounds demonstrated a rapid bactericidal mode of action against both Gram-positive and Gram-negative species. The mechanism of action of this type of compound was explored both *in silico* and through a range of microbiological techniques. These methods suggest that inhibition of DNA gyrase is one of the main mechanisms of action, although the possibility of activity through other mechanisms cannot be completely ruled out. The efflux liability and entry into Gram-negative species of the chemical series and the likelihood of the emergence of resistance was also studied. Importantly, the relative lack of DNA binding, as observed by FRET-based DNA melting, combined with the absence of eukaryotic toxicity, highlights the potential therapeutic value of this new type of C8-linked pyrrolobenzodiazepine monomers as antibacterial compounds.

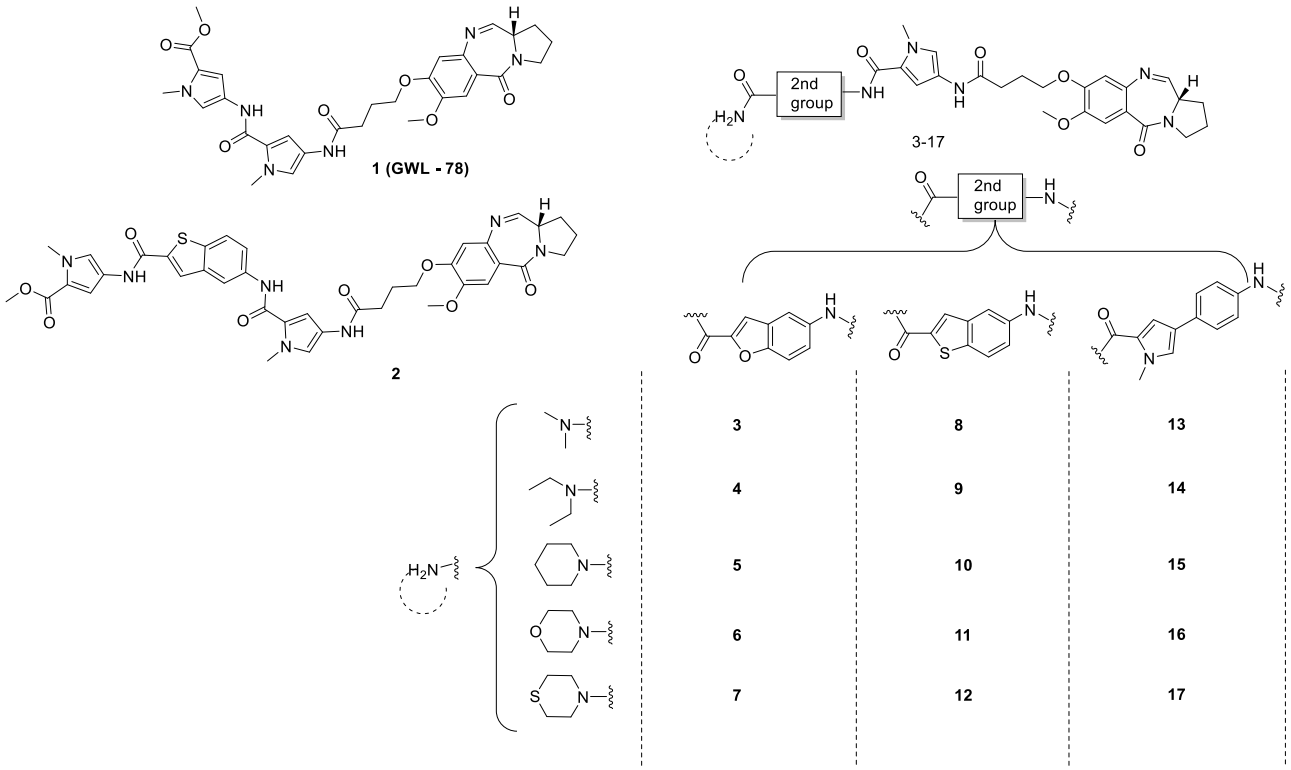


Figure 2: Structure of control compound **1**,**2** and structures of final compounds **3-17**

Results and Discussion

Design and Synthesis of PBD monomers

Three C8-linked biaryl-PBD monomers with reported activity against Gram-positive species were selected as the starting point for the medicinal chemistry modification (Figure 1). These monomers contain C8-substituents with two heteroaromatic or biaryl building blocks connected via amide bonds. The side chains were elongated with a third aliphatic heterocycle resulting in an unconventional class of PBD derivatives that have never been investigated before. Initially, three nitrogen-containing aliphatic heterocycles, thiomorpholine, morpholine and piperidine, were introduced and this was followed by coupling the heteroaromatic ring containing PBD monomers with diethyl and dimethylamine. The structural modification aimed to modify the physicochemical properties of the molecules and occupy a different chemical space as polar compounds have traditionally shown better activity against Gram-negative bacteria[17]. The secondary aim was to reduce the DNA binding affinity of the compounds to minimise eukaryotic toxicity, as the replacement of an aromatic group with an aliphatic ring was expected to reduce both interaction and

fit of the molecules within the DNA minor groove. A total of 15 target compounds were designed and successfully synthesised to explore the structure-activity relationship (SAR) (Figure 2) and understand the role of the third ring in extending the antibacterial activity of the compounds. C8-linked monomers **1** (GWL-78) and **2,** in which the heteroaromatic pyrrole ring replaced the third aliphatic ring, were used as control to compare their activity against Gram-negative bacteria.

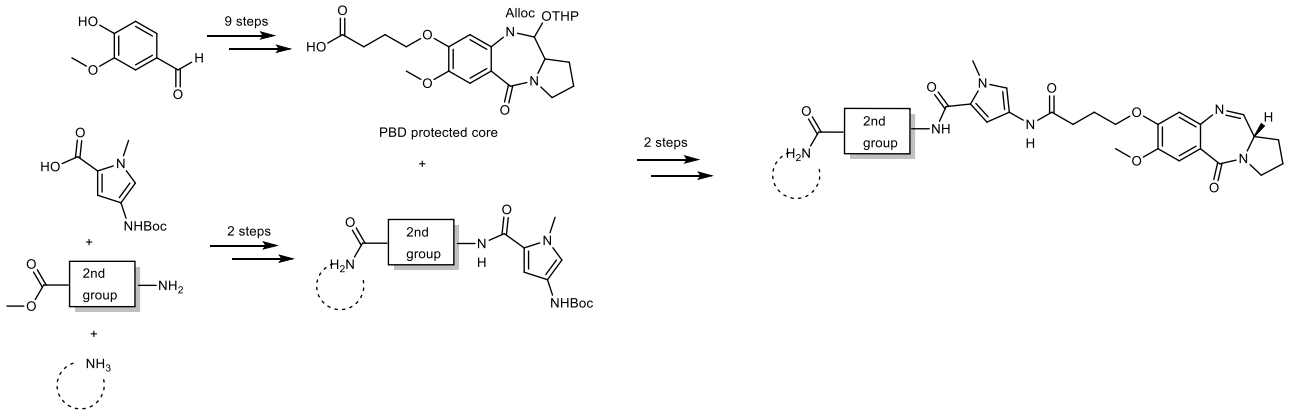
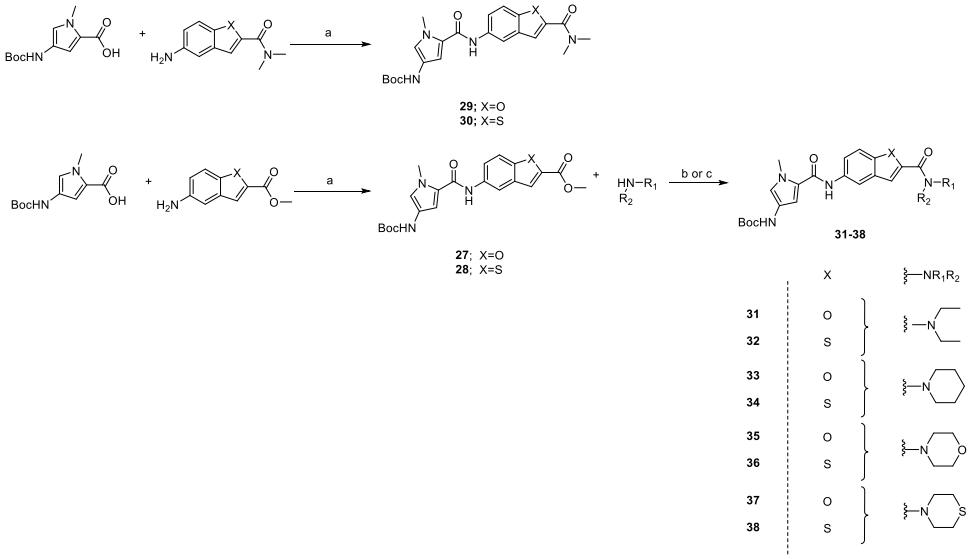


Figure 3: Synthetic approach to obtain antibacterial PBD monomers

The target compounds were synthesised using a convergent strategy based on literature reported procedures. The synthetic process is based on the C8 derivatization of an alloc, THP-protected pyrrolobenzodiazepine core with different polyamide side chains. The protected PBD core **26** was synthesised in nine steps starting from vanillin accordingly to a reported procedure from our group. The synthetic methods and characterization of intermediates that lead to **26** are included in the supporting information file (chemistry section). The three-component side chains were synthesised by sequential addition of the different constituents via amide coupling. Alternative strategies based either on the use of protecting groups or functional groups interconversion, were employed to avoid side reactions. For the synthesis of benzofused containing lateral chains (scheme 1) the initial step involved the formation of an amide bond between Boc-protected N-methyl pyrrole carboxylic acid and the corresponding 5-amino benzofused methyl ester to give intermediates **27** and **28**. The esters derivative underwent basic hydrolysis to give the carboxylic acids that were successfully coupled with diethylamine, piperidine, morpholine or thiomorpholine to give the complete C8-side chains **31-38**. The coupling system EDCI/DMAP was used as reagent for the formation of the amide bonds with good yield with the only exception of diethylamine products. In this case the coupling between the carboxylic acid derivatives of **27** and **28** and the secondary amine was performed using a

propylphosphonic anhydride (T_3P) sustained dehydration reaction in the presence of DIPEA. The synthesis of dimethylamine derivative **29** and **30** was accomplished with direct coupling between Boc-protected N-methyl pyrrole carboxylic acid and corresponding commercially available 5-amino benzofused dimethyl carboxamides. The coupling system EDCI/DMAP was used for formation of the amide bond similarly to previous examples.

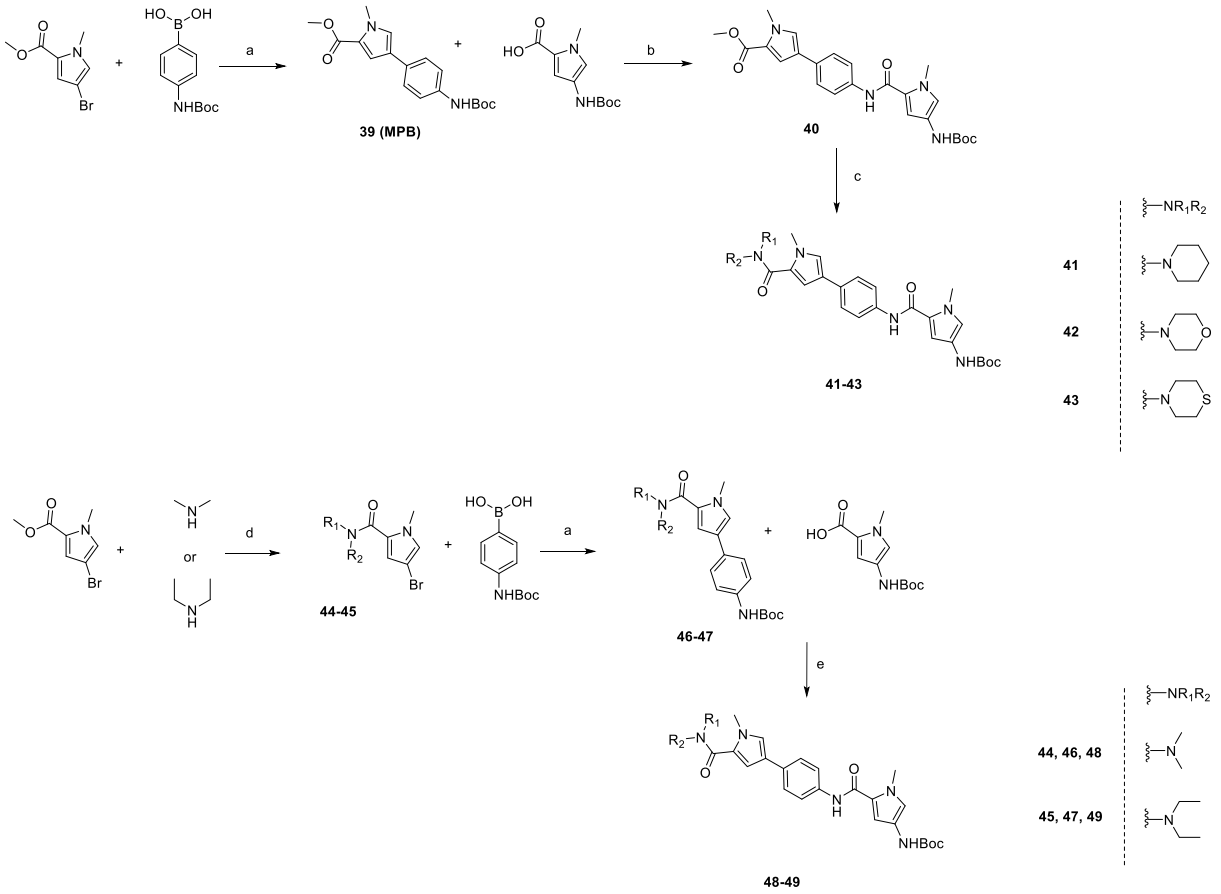


Scheme 1: Synthesis of C8 benzofused lateral chains **29-38**. Reagents and conditions: a) EDCI, DMAP, DMF, r.t., overnight; b) NaOH, MeOH, H_2O, r.t., overnight then EDCI, DMAP, DMF, r.t., overnight; c) NaOH, MeOH, H_20, r.t., overnight then T_3P, DIPEA, DMF, r.t., overnight

Two different approaches were employed, depending on the aliphatic substitution of the products, for the synthesis of methylpyrrole benzenamine (MPB) containing lateral chains (scheme 2). In the case of piperidine, morpholine and thiomorpholine substitution, the MPB central building block **39** was synthesized via Suzuki coupling between Br-methyl pyrrole carboxylic acid and the para-Boc-protected benzenamine boronic acid. After acid catalysed Boc-deprotection, the aniline derivative of **39** underwent amide coupling reaction with Boc-protected N-methyl pyrrole carboxylic acid. The reaction was performed with EDCI/DMAP coupling system to give intermediate **40**. The synthetic process was completed by basic hydrolysis of the methyl ester moiety and successive EDCI/DMAP sustained amide coupling reaction with piperidine, morpholine or thiomorpholine to give complete side chain **41-43** respectively. This synthetic approach failed in the formation of dimethyl and diethyl amide derivatives due to the poor yield of amide coupling reaction of the considered secondary amines using traditional coupling reagents.

For this reason, a reverse-direction synthetic process, with aliphatic derivatization as the first step, was applied for the synthesis of dimethyl and diethyl substituted MPB containing side chains. Specifically, the synthetic route was modified with the initial synthesis of dimethyl and diethyl N-methyl Br pyrrole intermediates **44** and **45** via amide bond formation based on acyl chloride formation. Commercially available 4-Br-N-methyl pyrrole methyl ester was hydrolysed and the corresponding carboxylic acid was activated to acyl chloride using oxalyl chloride in the presence of catalytic DMF. The reaction mixture was then treated with the corresponding secondary amine to give the desired product.

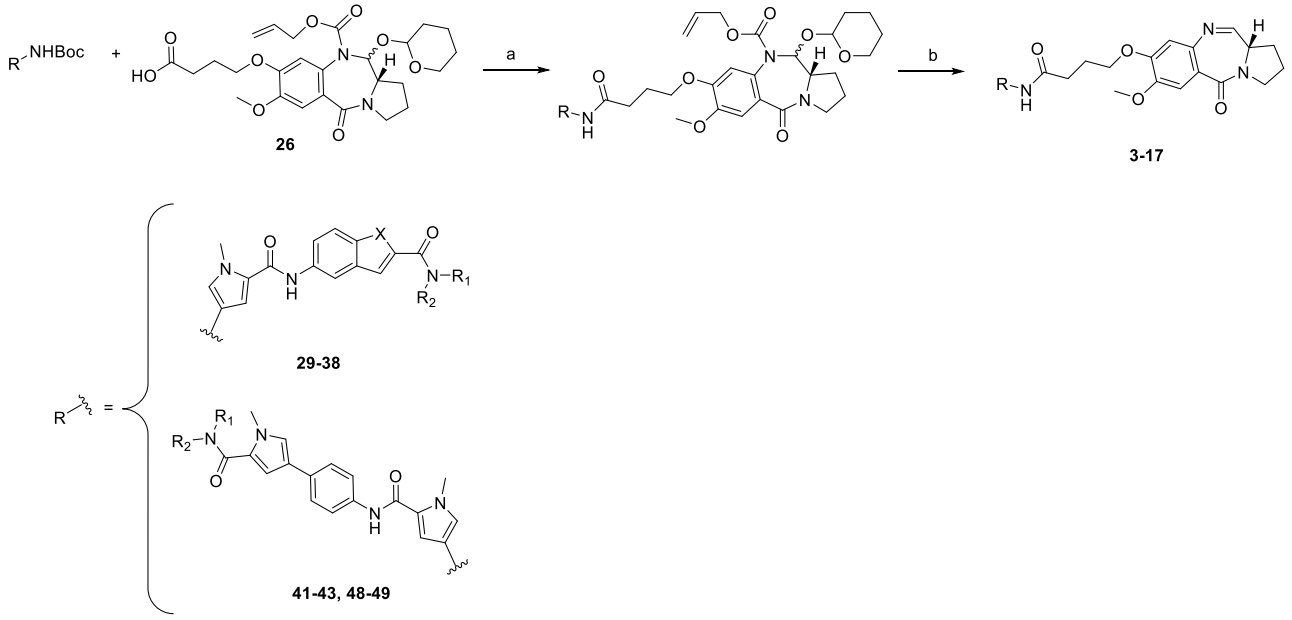
Intermediates **44** and **45** underwent Suzuki coupling reaction with para-Boc-protected benzenamine boronic acid to give MPB modified derivatives **46** and **47**. The obtained intermediates were deprotected via acid treatment and then successfully coupled with Boc-protected N-methyl pyrrole carboxylic acid to give final complete lateral chains **48**-**49**.



Scheme 2: Synthesis of C8 MPB containing lateral chains **41**-**43**, **49**-**49**. Reagents and conditions: a) Tetrakis(triphenylphospine)Pd, K_2CO_3, ACN, H_2O, MW irradiation, 100 °C, 6 minutes; b) EDCI,

DMAP, DMF, r.t., overnight; c) NaOH, MeOH, H_2O, r.t., overnight then EDCI, DMAP, DMF, r.t., overnight; d) oxalyl chloride, DMF, dry DCM, Et_3N, r.t., overnight; e) HCl 4M in dioxane, MeOH, r.t., 30 minutes then EDCI, DMAP, DMF, r.t., overnight.

Finally, the convergent synthetic strategy was completed with the coupling of C8-side chains **29**-**38**, **41**-**43**, **48**-**49** to the PBD core **26** using EDCI/DMAP mediated amide coupling. This reaction was followed by palladium catalysed deprotection of allyl carbamate moiety and concerted THP deprotection of the alchool moiety to generate the reactive N10-C11 imine bond, giving the final products **3-17** (scheme 3).



Scheme 3: Synthesis of PBD monomer **3-17**. Reagents and conditions: a) HCl 4M in dioxane, MeOH, r.t., 30 minutes then EDCI, DMAP, DMF, r.t., overnight; b) Tetrakis(triphenylphospine)Pd, piperidine, triphenylphospine, DCM, r.t., 30 minutes.

Screening of PBDs to define SAR

Bacterial panels containing drug sensitive and MDR strains of *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus spp.* were used as an initial screen to determine the efficacy of the PBD compounds in this series and establish a structure-activity relationship (SAR) (Tables 1 and 2) (resistance profiles for all strains are in Table S1). All the designed PBD molecules had a 3rd aliphatic ring or a ring fragment and contained pyrrole as the first unit followed by methylpyrrole benzenamine (MPB), benzofuran or benzothiophene as the second unit. Against the Gram-negative species tested, the control PBD

molecule **2** did not show any notable activity, with minimum inhibitory concentrations (MICs) ranging from 64 to >128 mg/L (Table 1). However, the aliphatic 3rd ring containing C8-linked PBDs (compounds **3 - 17**) showed notable improvement in activity against Gram-negative bacteria, except *P. aeruginosa*, with MICs as low as 0.5 mg/L against MDR *A. baumannii* and *K. pneumoniae* strains.

Table 1: Activity of the synthesized compounds against Gram-negative bacteria (mg/L)

	A. baumannii		***K. pneumoniae***		***P. aeruginosa***	
Resistance profile	**S**	**MDR**	**S**	**MDR**	**S**	**MDR**
Compound	**ATCC 17978**	**AYE**	**M6**	**NCTC 13368**	**PA01**	**NCTC 13437**
1	128	128	>128	>128	128	>128
2	64	64	128	128	>128	128
3	4	4	0.5	16	128	64
4	4	4	8	64	128	32
5	2	2	8	64	128	32
6	8	8	0.5-1	32-64	>128	64
7	2	2	1	32	>32	>32
8	1	0.5	1	2	>32	>32
9	2	2	8	64	128	32
10	8	8	128	128	128	64
11	8	8	64	8	64	32
12	2	2	>128	2	64	32
13	4	4	2	8	128	>128
14	64	32	>128	>128	>128	>128
15	2	2	16	>128	>128	>128
16	4	2	2	32	>128	>128
17	2	2	4	>32	>32	>32

Compounds bearing GC-targeting the methylphenylbenzenamine (MPB) substituent as a central group (compounds **13, 15** to **17**) showed good efficacy against *A. baumannii* and *K. pneumoniae* but were inactive against *P. aeruginosa*. Compounds containing benzofuran as the second ring were

found to be more active than the corresponding benzothiophene series. The structural modification of the 3rd aliphatic ring showed a significant influence on the activity of the compounds. In all cases compounds with thiomorpholine with the third ring (example compounds **7**, **12** and **17**) had MICs of ≤ 2 mg/L against the *A. baumannii* strains tested. The presence of a second heteroatom in the aliphatic 3rd ring improved activity, but intriguingly, shortening the 3rd ring to a dimethyl or diethyl side chain (example compounds **3**, **4**, **8**, **9** and **13**) retained activity, with dimethyl analogues showing better activity than their diethyl counterparts, except compounds containing the MPB ring in which diethyl substitution, compound **14**, resulted in loss of activity.

Against the Gram-positive species tested, all compounds, including the control PBD molecule, showed excellent activity, with MICs ranging from 0.03 to 1 mg/L (Table 2).

Table 2: Activity of the synthesized compounds against MDR Gram-positive bacteria. MICs are expressed in mg/L.

	E. faecium	***E. faecalis***		***S. aureus***		
Resistance profiles	**MDR (VRE)**		**S (VSE)**	**MDR (MRSA)**		**S (MSSA)**
Compound	**NCTC 12204**	**NCTC 12201**	**NCTC 775**	**NCTC 13616**	**NCTC 13277**	**NCTC 6571**
1	0.5	0.5	1	1	2	0.5
2	<0.125	<0.125	<0.125	0.5	1	<0.125
3	<0.125	0.5	<0.125	<0.125	<0.125	<0.125
4	<0.125	<0.125	0.25	0.25	0.5	<0.125
5	<0.125	<0.125	0.25	0.25	0.5	<0.125
6	<0.125	<0.125	<0.125	<0.125	0.25	<0.125
7	<0.125	<0.125	<0.125	<0.125	0.25	<0.125
8	0.03	0.03	0.03	0.03	0.06	0.03
9	1	0.5	1	1	1	0.5
10	<0.125	<0.125	0.25	0.5	1	<0.125
11	<0.125	<0.125	<0.125	<0.125	0.5	<0.125
12	<0.125	<0.125	<0.125	<0.125	<0.125	<0.125
13	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12
14	0.25	0.25-0.5	0.5	1	1	0.25-0.5
15	<0.03	<0.03	0.12	0.06	0.12	<0.03
16	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12
17	<0.03	<0.03	<0.03	<0.03	0.06	<0.03

Two compounds (**7** and **8**) with promising activity against both Gram-negative and Gram-positive strains were selected for further screening against a wider panel of bacteria including MDR Gram-negative strains of *E. coli, A. baumannii, K. pneumoniae* and *Burkholderia cepacia/cenocepacia* complex. The *K. pneumoniae* strains selected for the study were resistant against most marketed antibiotics (Table 3 and Table S1) including one essentially PDR strain (51851). Similarly, the *A. baumannii* strains and *E. coli* strains containing ESBL and carbapenemase genes were resistant to 6 to 8 clinically used antibiotics (Table S1). Both compounds were found to be active against almost all strains tested except *B. multivorans* C1962 against which compound **8** was found to be inactive (defined here as an MIC >32 mg/L). The levels of activity observed, considering the high levels of intrinsic or acquired resistance in many of these strains, are exceptional, with low and in some cases sub µM MICs. Notably, compound **7** exhibited a range of MICs from 0.25 to 2 mg/L for *K. pneumoniae*, 0.125 to 1 mg/L for *A. baumannii/baylyi*, 0.5 to 1 mg/L for *E. coli* and 0.125 to 2 mg/L for *B. cepacia* complex. These values are exciting and suggest that this represents a promising new chemical scaffold that can be further developed as broad-spectrum antibiotics.

Table 3: Activity of compounds **7** and **8** against an extended panel of MDR Gram-negative bacteria (MIC in mg/L).

Species	Strain	**7**	**8**
		MIC	
K. pneumoniae	NCTC 13368	2	32
	M6	1	1
	NCTC 13438	0.5	4-8
	NCTC 13439	2	4-8
	NCTC 13443	2	16
	16	0.25	1
	46704	1	4
	51851	2	4
	MGH 78578	1	4-8
A. baumannii	ATCC 17978	0.5	2
	AYE	1	2
	NCTC 13424	0.5	1
	ADP1	0.125	1
	NCTC 13302	0.25	4
	UKA2	0.5	4
	UKA7	0.5	4
	W1	1	4
E. coli	CFI_017_VIM4	1	2
	CFI_033_NDM	0.5	1
	CFI_160_KPC2	0.5	4
	CFI_161_NDM_5_OXA_181	0.5	1
	LEC001	1	2
	319238/UR	0.5	1
	NCTC 12923	0.5	2
B. multivorans	C1576	1	16
	C1962	2	>32
B. cenocepacia	K56-2	2	1
B. cepacia	CEP509	2	8
	NCTC 17765	0.125	1
	LMG 17997	0.125	0.25
	NCTC 10743	1-2	16

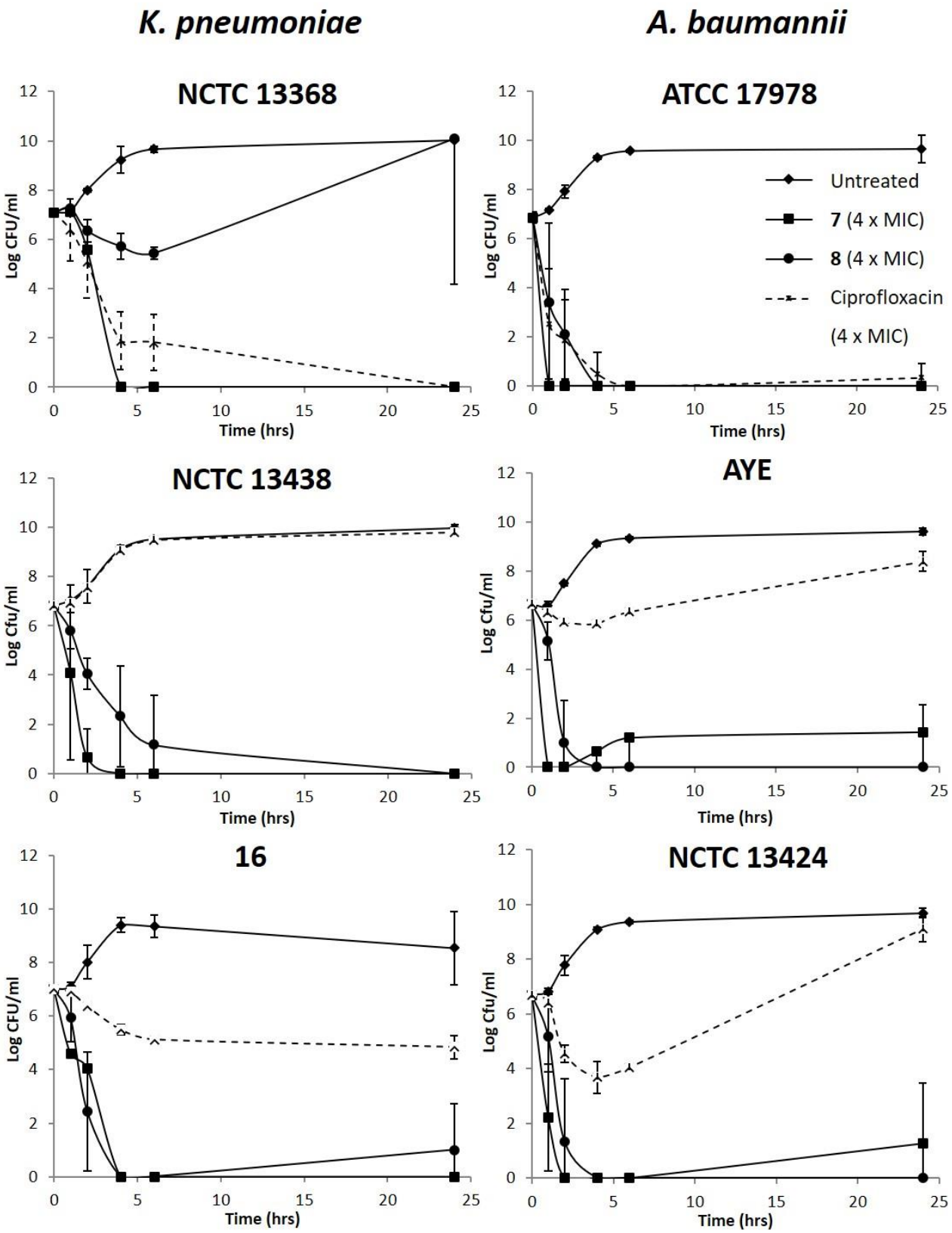


Figure 4: Rapid bactericidal kill by synthesised compounds **7** and **8**, with occasional breakthrough resistance in some drug-strain combinations. The data are the average of three independent experiments and error bars represent the standard deviation from the mean.

Defining the mode of action of PBDs through time-kill assays

The mode of action of compounds **7** and **8** was explored using time-kill assays. Three *K. pneumoniae* (NCTC 13368, 16 and NCTC 13438) and three *A. baumannii* (AYE, ATCC 17978 and NCTC 13424) strains were selected for time-kill analysis (Figure 4). These strains were selected to provide a range of drug resistance profiles from a relatively drug-sensitive strain (ATCC 17978), through to MDR strains with multiple resistance mechanisms (NCTC 13438, 16 and NCTC 13424) for each species. Compound **8** was rapidly bactericidal in all strains tested; the viable count was below the limit of detection (LOD) within 4 hours and continued to be below the LOD for the full assay for all *K. pneumoniae* strains challenged with **8,** indicating that there was no resistance to this compound within the 24 hour period of the assay. A small number of viable cells were observed at the end of 24 hours for *A. baumannii* strains AYE and NCTC 13424 (Figure 4). Subsequent testing with **8** on these populations showed no increase in MIC above wild-type levels, and these populations were not considered to be resistant (results not shown). Compound **7** was also bactericidal against the majority of strains tested, although the speed of killing was slower than compound **8** in a number of cases (NCTC 13438, AYE, NCTC 13424). In the case of *K. pneumoniae* NCTC 13368, which had a much higher intrinsic resistance to compound **7**, only a small reduction in viable count was observed at 6 hours, with bacterial numbers returning to the levels of the untreated control by 24 hours, in two out of three replicate experiments. When these bacteria were isolated, passaged 10x in the absence of selection and re-assayed, the MICs increased for both compound **7** (32 to ≥128 mg/L) and compound **8** (2 to >32 mg/L). No increases in MIC were observed for other antibiotics, including aminoglycosides, fluoroquinolones and β-lactams, except for an increase in colistin resistance of 4 to 32mg/L in one of the two isolates (Table S2).

Evaluation of the influence of efflux and influx on the activity of the PBD compounds

Two efflux pump inhibitors, PAβN (used in the presence of Mg^{2+} to prevent non-specific effects on membrane permeability) [18] which competitively inhibits specific RND efflux pumps and CCCP, which inhibits the proton motive force, were used to assess the impact of efflux on the activity of the lead compounds against wild-type bacterial strains, together with the resistant mutants isolated from the time-kill experiments.

The result (Table 4) suggests that both compounds are substrates for RND-family efflux pumps, but the presence and/or expression of specific efflux pumps differs between the strains tested. To investigate this further, specifically in the *P. aeruginosa* strain PAO1, transposon mutants in various RND efflux pumps were obtained from the Manoil collection (Univ. Washington; Table

S3). Transposon mutants in any component of the tripartite RND efflux system MexAB-OprM reduced the MIC by ≥8-fold for both compounds **7** and **8**. The MIC of compound **8** was also decreased ≥4 fold by transposons in MexCD but not its outer membrane component OprJ; no such effect was observed for compound **7** with transposon mutants in any component of this pump.

A membrane permeabiliser, polymyxin b nonapeptide (PMBN), used at a concentration which did not affect bacterial growth in its own right, was also used to understand to what extent efficacy for compounds with high MICs were impacted by entry of the compounds into the cell. Effects in *P. aeruginosa* were particularly marked, with addition of PMBN reducing the MIC of **8** against the MDR strain NCTC 13437 from >32 mg/L to 0.03 - 1.0 mg/L, a reduction of between 32- and 1000-fold. Similarly, a reduction in MIC against PAO1 was observed between 4- and ≥16-fold for compound **8**. PMBN also reduced the MIC of compound **7** against an extended panel of *P. aeruginosa* strains from >32 to 1 – 8 mg/L (8- to ≥64 fold). The activity of these compounds in *P. aeruginosa* strains in the presence of a permeabilizer shows that these compounds are effective at killing strains once they are able to cross the outer membrane barrier. This offers the opportunity to optimise the active compounds using medicinal-chemistry modifications or to explore active delivery mechanisms to allow the compounds to permeate the *Pseudomonas* membrane.

Table 4: Inhibiting efflux and increasing influx potential reduces the MIC for selected compounds in strains of *K. pneumoniae*, *A. baumannii* and *P. aeruginosa*. A four-fold change in MIC in the presence of efflux inhibitors or membrane permeabilisers was defined as significant (highlighted by shading). All values are given in mg/L. These data are representative of three or more independent experiments. ND = Not done.

				Efflux inhibition		Outer membrane permeabilisation
	Strain	Compound	MIC	CCCP	PAβN	PMBN
Klebsiella	NCTC 13368	**8**	2	2	0.5	ND
		7	32	>32	8	8
	NCTC 13368-**7**-R1	**7**	128	128	32	8
	NCTC 13368-**7**-R2	**7**	>128	>128	128	64
	16	**8**	1	0.06-0.5	2	0.25
		7	1	0.12-1	2	0.5
	NCTC 13438	**8**	0.5	0.5	0.25	ND
		7	4	4	4	ND
Acinetobacter	AYE	**8**	1	0.125	0.25	ND
		7	4	4	2	ND
	ATCC 17978	**8**	0.5	0.25	0.25	ND
		7	2	2	2	ND
	NCTC 13424	**8**	0.5	0.25	0.25	ND
		7	2	1	2	ND
Pseudomonas	PA01	**8**	>32	>32	2	2-16
		7	128	>32	32	0.25-8
	NCTC 13437	**8**	>32	>32	16	<0.03-1
		7	>32	>32	>32	2-32

Understanding the mechanism of action.

Effect of the aliphatic third ring substitution on DNA binding and eukaryotic toxicity of the synthesized compounds.

The cytotoxicity of C8-linked PBD monomers is linked with their ability to stabilise DNA sequences. A FRET-based DNA melting assay[7] was used to explore the effect of the aliphatic third ring on the DNA stabilisation property of the newly synthesized molecules. Two fluorophore labelled oligonucleotides (Sequence F1: 5'-FAM-TAT-ATA-TAG-ATA-TTT-TTT-TAT-CTA-

TAT-ATA-3'-TAMRA, and Sequence-F2: 5'-FAM-TAT-AGA-TAT-AGA-TAT-TTT-ATA-TCT-ATA-TCT-ATA-3'-TAMRA) with preferred PBD binding sites were used in the assay, and the stabilisation was compared with a previously reported C8-PBD monomer; GWL-78[19] (compound **1**) and the control compound **2** with the third heteroaromatic ring. In all cases, compounds with aliphatic 3rd moiety showed between 3- to 4-fold lower ΔTm values at both 5:1 and 1:1 ligand-DNA ratio compared to the control compounds, showing that the introduction of the aliphatic 3rd moiety reduced the ability of these compounds to interact with and stabilize the DNA sequences (Table S4). The eukaryotic toxicity of the synthesised PBD derivatives was tested at a single concentration (20 μM) using the MTT viability assay on WI-38 fibroblast cell line. The control compounds, **1** and **2**, showed potent cytotoxicity with only 35% and 32% of cells viable after 24 hours. However, the newly synthesized compounds showed notably less toxicity, with >85% of cells viable after 24 hours treatment with the molecules (Figure S1). Considering an average molecular weight of 700 Da for the synthesized PBD monomers, the concentration used for the test (20 μM) corresponds to a concentration of 14 mg/L, which is approximately 100 times the average MIC reported for Gram-positive strains and almost 10 times the average MIC reported for Gram-negative strains for active compounds. The obtained results confirmed the presence of a selective toxicity profile towards prokaryotic cells for the PBD-C8 conjugates, confirming the possibility of further development of this class of compounds as antibacterial agents.

Inhibition of DNA Gyrase

The C8-linked PBDs have been recently reported as DNA gyrase inhibitors[14]. This was further explored by docking compound **7** and **8** with the bacterial gyrase from *S. aureus* (PDB ID 2XCT) and E. coli (PDB ID 6RKS) (Figure 5 and Figure S2). Both compounds bound to the ligand binding domain of DNA gyrase A and showed interaction with several amino acid residues through hydrogen bonds and hydrophobic interactions. The best pose of compound **7** gave a ChemScore of 27.62 and a binding affinity of -31.67 kcal/mole, and the values for compound **8** was comparable with a ChemScore of 27.35 and binding affinity of – 30.70 kcal/mole in the case of DNA gyrase A from *S. aureus*. For the DNA gyrase A from *E. coli* the ChemScores were 25.16 and 22.01 while the free energy of binding values were 30.46 kcal/mole and -25.51 kcal/mole for compounds **7** and **8**, respectively. The 2D models shown in Figure S3 suggests compound **7** forms six hydrogen bonds within the ligand binding domain of DNA gyrase A from *S. aureus,* and five hydrogen bonds within the ligand binding domain of DNA gyrase A from *E. coli.* Similarly, compound **8** forms five hydrogen bonds within the ligand binding domain of DNA gyrase A from *S. aureus,* and four hydrogen bonds within the ligand binding domain of DNA gyrase A from *E. coli.* Both compounds show notable hydrophobic interaction within the binding site (Tables S5 to S8). The interaction of compounds **7** and **8** with the ligand binding sites of Gyrase A suggests the inhibition of Gyrase A plays a crucial role in the antimicrobial activity of this class of compound.

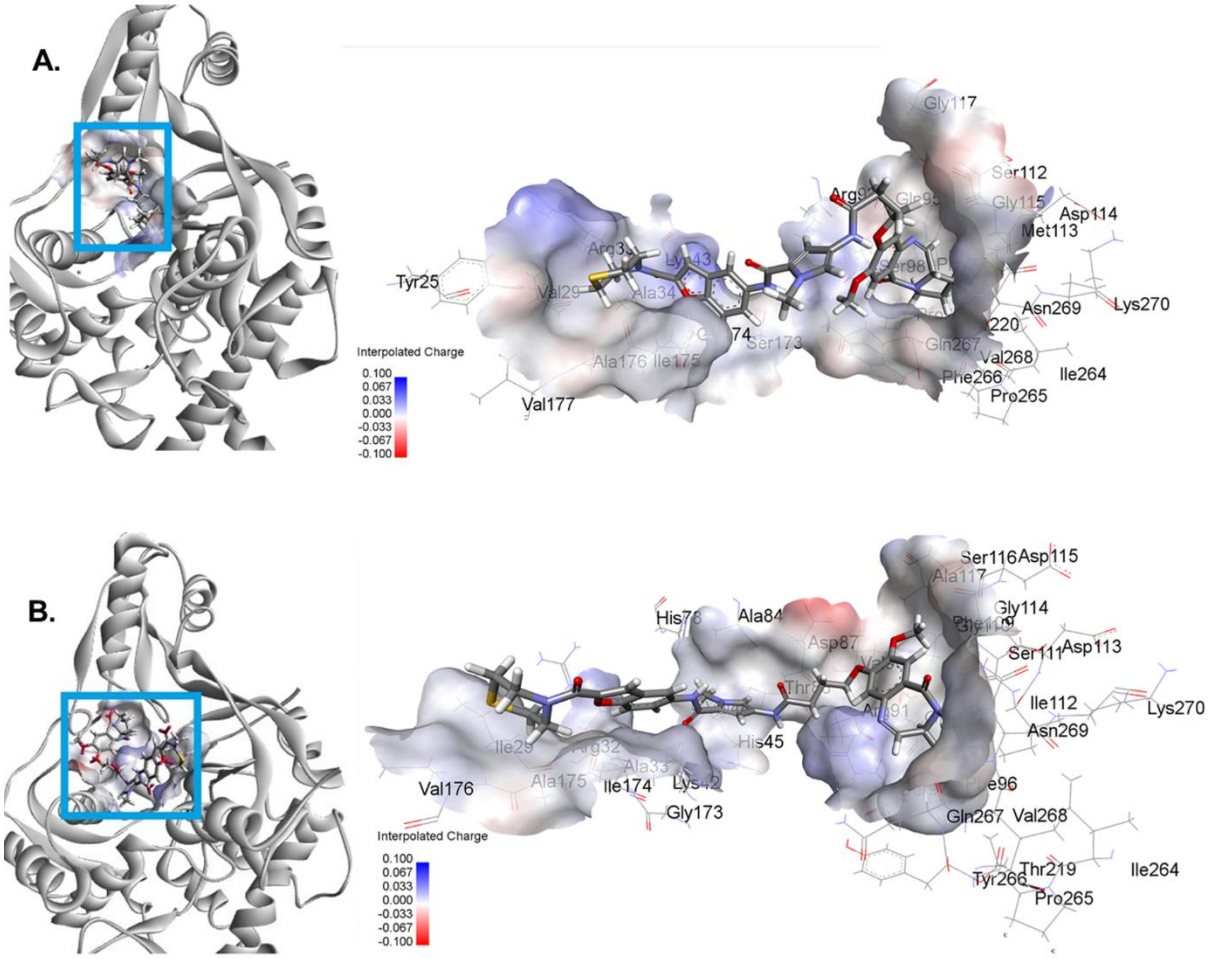


Figure 5: Molecular model showing the location of the binding pocket and the interaction of compound **7** with A) DNA gyrase A from *S. aureus*; **B)** DNA gyrase A from *E. Coli.*

We have recently shown that PBD monomers caused inhibition of DNA gyrase in a rapid assay format[14]. A gel based assay was used here to evaluate whether compounds **7** and **8** were also able to inhibit DNA gyrase *in vitro*. In assays using purified *S. aureus* DNA gyrase, compound **8** showed an IC50 of 3.16 ± 1.36 mg/L compared to ciprofloxacin IC50 of 13.05 ± 1.36 mg/L (Figure 6). It was not possible to determine an IC50 for compound **7** as solubility issues prevented the use of high enough concentrations of compound to achieve > 50% inhibition (at a concentration of 32 mg/L). Against the *E. coli* DNA gyrase it was not possible to reach a concentration high enough to achieve greater than 50% inhibition for either compound **7** or **8**, again due to solubility issues. The relative inhibition of the Gram-positive *vs.* Gram-negative gyrase correlates with the relatively higher MICs of Gram-negative bacteria species and this supports the idea that the novel PBDs described here work through inhibition of DNA gyrase.

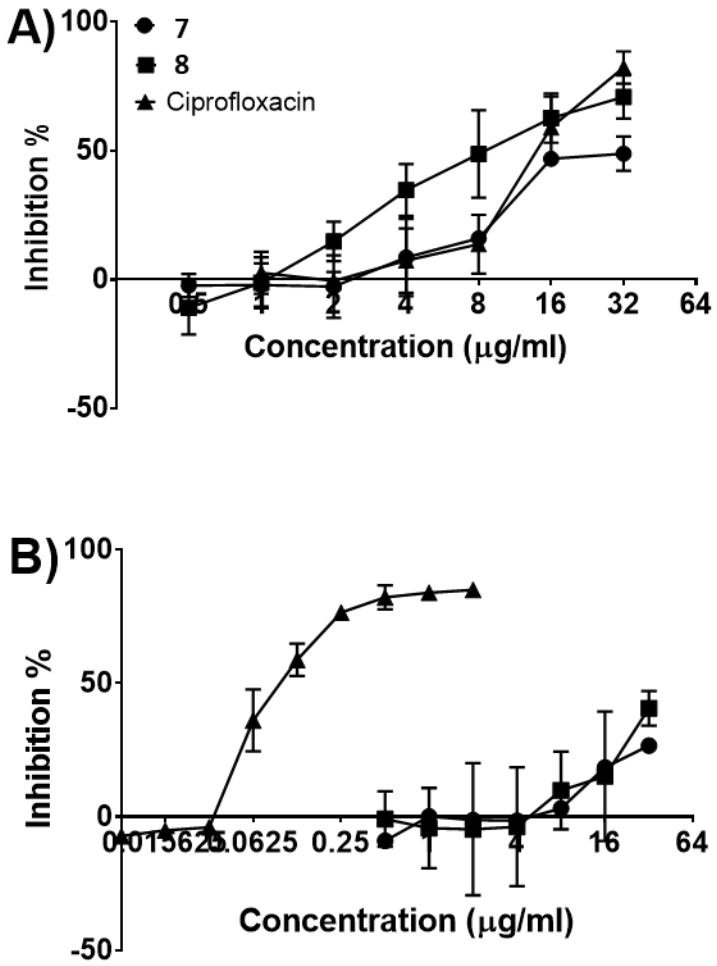


Figure 6: Inhibition of gyrase enzyme from A) *S. aureus* and B) *E. coli* by synthesized compounds **7** and **8** and the control, ciprofloxacin. Results represent the mean of three independent experiments, the error bars are the standard deviation of the mean.

Whilst we have evidence of inhibition of DNA gyrase activity, for both Gram-positive and Gram-negative DNA gyrase, this does not preclude the possibility that the PBDs cause inhibition via binding to DNA and/or a DNA-Gyrase complex. To further define the mechanism of action, a panel of fluorescent *E. coli* reporter strains were used to understand the profile of cellular responses to PBDs and compare these with molecules with known mechanisms of action. A panel of strains derived from a comprehensive library of *E. coli* promoter fusions were used [20]. Specific reporters for compounds affecting DNA metabolism were selected, in line with previous studies [21] and validated with DNA gyrase inhibitors (fluoroquinolones, novobiocin), compounds known to bind DNA (mitomycin C, PBD control) and control compounds affecting other cellular targets (doxycycline, ampicillin, rifampicin). The four promoter fusions showed the expected induction

with fluoroquinolones (specifically levofloxacin, ciprofloxacin, norfloxacin and moxifloxacin) and the quinolone antibiotic nalidixic acid, associated with activation of the bacterial SOS response as a result of the poisoning of the DNA gyrase. The level of fold induction was similar between the known gyrase inhibitors, with fold inductions ranging from 20-35 fold for *recA*, 5-13 fold for *lexA*, 14-32 fold for *recN* and 17-41 fold for *sulA* (Figure 7). Whilst compound **7** and **8** both showed activation of the same 4 promoter fusions, the levels were significantly lower for each of the genes (8-10 fold recA, 5-9 fold lexA, 6-11 fold recN and 6-7 fold sulA). By comparison, the three antibiotics known not to induce the SOS response, doxycycline, rifampicin and ampicillin, showed no induction of any of the four promoters above the level of the control strain. Interestingly, three compounds showed similar levels of activation of the promoters to the two PBDs. Two of these are known to be DNA binding molecules, mitomycin C and the control PBD dimer DSB-120[22], whilst the third, novobiocin, inhibits DNA gyrase by interaction with the ATP-binding site on the GyrB subunit. These results suggest that the PBDs act by interfering with DNA metabolism, in a way that is clearly different from fluoroquinolones. This is consistent with MIC data which shows no significant differences between fluoroquinolone sensitive and resistant strains. Further studies will be required to delineate the specific interactions with DNA and/or individual gyrase subunits and the specific mechanisms of inhibition.

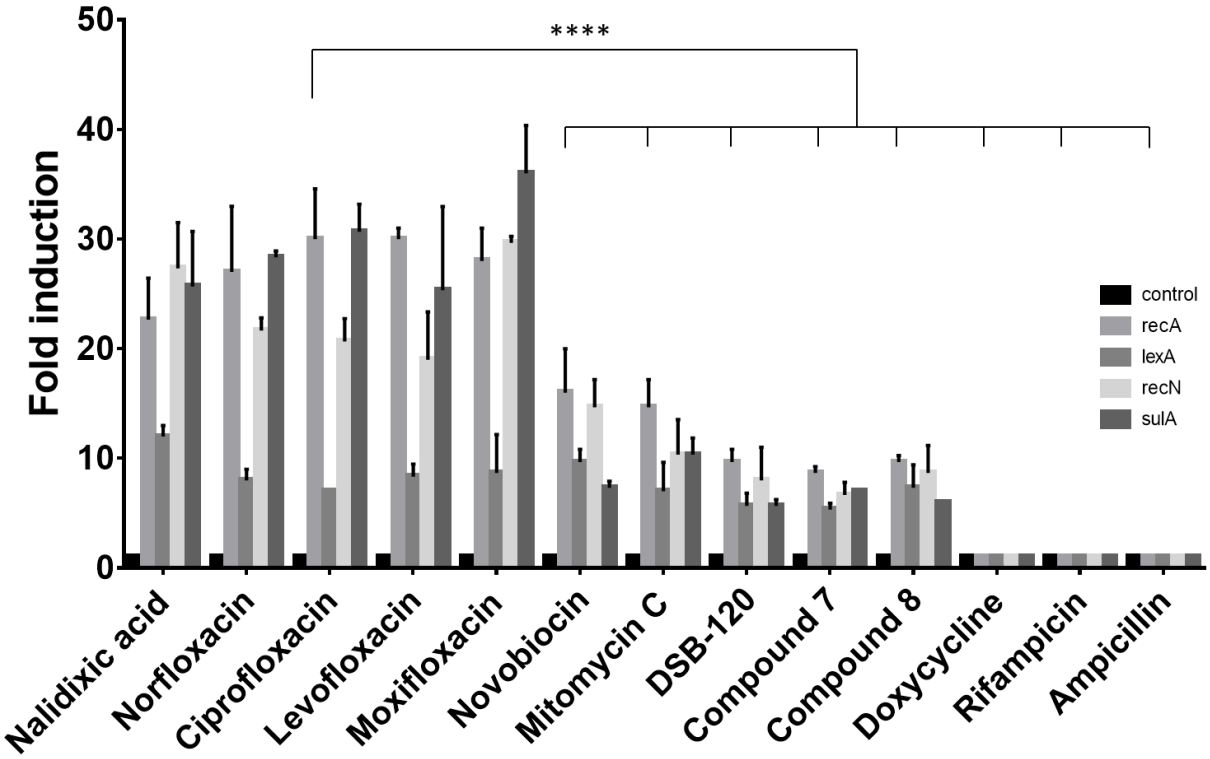


Figure 7: Reporter assay to explore the mechanism of action of compounds **7** and **8.** Data is the mean of three independent experiments and error bars represent the standard deviation of the mean. 2-way ANOVA and Dunnett's multiple comparisons test were performed and included here for reporter strain recA, all compounds compared to ciprofloxacin **** $p < 0.0001$.

Emergence of resistance – Mutation Frequency

The mutation frequency of the most active compound **8** was tested against representative strains of the three Gram-negative pathogens in comparison with ciprofloxacin. At 4 X MIC, mutation frequencies of $<4.3 \times 10^{-8}$ and $<1.5 \times 10^{-8}$ were measured across three replicate experiments for NCTC 12923 and ATCC 17978 respectively. In both cases, the values were significantly lower than the control antibiotic, ciprofloxacin (4.4×10^{-7} for NCTC 12923 and 8.4×10^{-7} for ATCC17978). This was not the case in the *K. pneumoniae* isolate M6, where the mutation frequency was 1.0×10^{-6} for compound **8,** compared to 8.9×10^{-7} for ciprofloxacin. This higher mutation frequency is reflected in the time-kill experiments, where resistance was only seen in a single *K. pneumoniae* strain (NCTC 13368), following exposure to concentrations above the MIC and not in *A. baumannii*.

The mutations associated with resistance in NCTC 13368 were identified by whole genome sequence analysis of stable resistant isolates. In one of the time-kill repeats, the resistant strains showed the deletion of a single base in position 50 of a gene encoding a predicted nucleoside-specific channel-forming protein (KPN_RS01945), termed Tsx. The mutation caused a frameshift in the *tsx* gene and was predicted to result in a truncated 42 amino acid protein. A second SNP was also identified in another uncharacterised permease gene, leading to an amino acid change M137K. A clean transposon mutant (tnkp1_lr150124p14q189) in *tsx*, in *K. pneumoniae* strain MKP103, also showed elevated resistance to compounds **7** and **8**, with respect to their parental strain (compound **7** from 32 mg/L in wild-type to >128 mg/L for *tsx* transposon, and for compound **8** from 16 to >32 mg/L) (Table S3). The Tsx protein has previously been described as mediating resistance to the antibiotic albicidin in *K. oxytoca*. [23]. A second resistant NCTC 13368 isolate showed a SNP, leading to an amino acid change H50N in a predicted MerR family regulator (KPN_RS12075).

Conclusions:

The lack of new antibiotics, especially for rapidly emerging MDR Gram-negative pathogens, is a significant cause for concern. Several studies have suggested that there may be no new antimicrobials, with distinct modes of action, available for treatment of these pathogens for a considerable time[24]. Against this background, we report the identification of a new scaffold with significant activity against both Gram-positive and MDR Gram-negative pathogens, which do not appear to be impacted by common resistance mechanisms to other broad-spectrum antibiotics and with a low mutation frequency leading to resistance in key species such as *A. baumannii* and *E. coli*.

Mechanistic evaluation shows a sub-micromolar IC50 in a gel-based *S. aureus* DNA Gyrase assay, significantly lower than ciprofloxacin and not affected by GyrA mutations associated with fluoroquinolone resistance. Using a panel of fluorescent reporters, it was possible to show activation of genes which are associated with the SOS response in Gram-negative bacteria, but again the magnitude of promoter activation differed from fluoroquinolones and was consistent with both known DNA binders (e.g. mitomycin C) and DNA gyrase inhibitors that act via alternative binding sites to fluoroquinolones (e.g. novobiocin).

The compounds show excellent activity against WHO priority pathogens with an *in vitro* selective index which would allow their continued development. The developability assessment of the lead compounds will be carried out to fully explore the clinical utility of this chemical class. There is renewed interest in developing bacteria specific antibody drug conjugates. We intend to exploit

well-understood and clinically validated routes to develop antibody drug conjugates (ADCs), which has been successfully employed for PBD-type compounds in oncology, to further improve the prokaryotic selectivity of these compounds and to provide selective targeting of such compounds to bacteria. This will form the basis of broad-spectrum PBD antibiotics which may have significant utility in the clinic.

Experimental Section

General chemistry

All reagents and solvents employed in the synthetic processes were obtained from commercially available sources including, among others, Sigma-Aldrich, Fisher Scientific, Fluorochem and Alfa Aesar. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) analyses were performed on a Bruker Spectrospin 400Hz spectrometer. LC-MS analyses were performed on a Waters Alliance 2695 system, eluting in gradient. The analyses were performed on a Monolithic C18 50 X 4.60 mm column by Phenomenex. UV detection was performed on a Diode Array Detector. Mass spectra were registered in both ESI+ and ESI- modes. Melting points were determined using a Stuart SMP30 melting point apparatus. All the compounds tested for their biological activity are >95% pure, confirmed with two different HPLC analysis methods The HRMS analyses were performed on a Thermo Scientific Exactive HCD Orbitrap Mass Spectrometer. The hydrogenation reaction was conducted using a Parr hydrogenation system. Synthetic protocols and compounds characterization for the previously reported PBD core are reported in supporting information file.

Synthesis of pyrrole benzofused intermediate 27 and 28

Commercially available 4-((tert-butoxycarbonyl)amino)-1-methyl-1H-pyrrole-2-carboxylic acid (200 mg, 0.8 mmol, 1.2 equiv.) was dissolved in DMF (5 mL). EDCI (2.5 equiv.) and DMAP (3 equiv.) were added to the solution that was left under magnetic stirrer in N_2 atmosphere for 20 minutes. At that point, the corresponding 5-amino-2-methyl ester benzofused (1 equiv.) was added to the reaction mixture and left under magnetic stirrer overnight. The reaction did not go to completion. The reaction was quenched by addition of water (15 mL) that was then extracted with ethyl acetate (3 x 10 mL). The organic phase was then sequentially washed with brine (15 mL), $NaHCO_3$ saturated aqueous solution (15 mL) and citric acid aqueous solution 0.1 M (15 mL). The collected organic phase was dried on MgSO4 and subsequently evaporated using a rotary evaporator giving crude of reaction that was subsequently purified by column chromatography (mobile phase: from 100 DCM to 85/15, v/v, DCM/EtOAc) to give the final products **27** and **28**.

6-(4-((tert-butoxycarbonyl)amino)-1-methyl-1H-pyrrole-2-carboxamido)benzofuran-2-carboxylate (27)

Obtained 0.200 g (reaction yield: 70%) as a yellow solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 8.09 (d, *J* = 2.01 Hz, 1H), 7.85 (s, 1H), 7.51 (d, *J* = 9.06 Hz, 1H), 7.47 (s, 1H), 7.40 (dd, *J* = 9.06 Hz, 2.27 Hz, 1H), 6.86 (br. s., 1H), 6.70 (br. s., 1H), 6.43 (br. s., 1H), 3.97 (s, 3 H), 3.91 (s, 3 H), 1.51 (s, 9H). ^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ159.9, 159.7, 152.5, 146.2, 134.0, 127.4, 123.3, 121.9, 121.3, 118.8, 114.2, 113.8, 112.6, 103.9, 80.4, 52.5, 36.8, 28.4. m/z (+ESI) calc. for $C_{21}H_{23}N_3O_6$ $(M)^+$ 413.1 found 414.0 $([M]+H)^+$.

Methyl 5-(4-((tert-butoxycarbonyl)amino)-1-methyl-1H-pyrrole-2-carboxamido) benzo[b]thiophene-2-carboxylate (28)

Obtained 0.192 g (reaction yield: 65%) as a yellow-orange solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ8.24 (d, *J* = 1.76 Hz, 1H), 7.97 (s, 1H), 7.78-7.76 (m, 2H), 7.49 (dd, *J* = 8.69 Hz, 2.14 Hz, 1H), 6.86 (br. s., 1H), 6.70 (br. s., 1H), 6.33 (br. s., 1H), 3.94 (s, 3H), 3.91 (s, 3H), 1.52 (s, 9H). ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ 163.2, 159.7, 153.5, 139.3, 137.7, 135.5, 134.4, 130.5, 123.3, 123.0, 122.0, 120.6, 119.0, 115.9, 104.1, 80.4, 52.5, 36.7, 28.3. m/z (+ESI) calc. for $C_{21}H_{23}N_3O_5S$ $(M)^+$ 429.1 found 430.0 $([M]+H)^+$.

Tert-Butyl (5-((2-(dimethylcarbamoyl)benzofuran-5-yl)carbamoyl)-1-methyl-1H-pyrrol-3-yl)carbamate (29)

Obtained 0.190 g (reaction yield: 72%), as a yellow solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ7.99 (d, J = 2.01 Hz, 1H), 7.70 (s, 1H), 7.48 (d, J = 8.81 Hz, 1H), 7.38 (dd, J = 8.94 Hz, 2.14 Hz,1H), 7.25 (d, J = 0.76 Hz, 1H), 6.88 (s, 1H), 6.68 (s, 1H), 6.32 (s, 1H), 3.92 (s, 3H), 3.35 (br. s., 3H), 3.15 (br. s., 3H), 1.52 (s, 9H). ^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ160.9, 159.8, 153.4, 151.5, 150.0, 133.8, 127.5, 123.4, 121.9, 120.0, 118.7, 113.5, 112.1, 111.9, 103.8, 80.4, 36.8, 28.4.m/z (+ESI) calc. for $C_{22}H_{26}N_4O_5$ $(M)^+$ 426.1 found 427.1 $([M]+H)^+$.

Tert-butyl (5-((2-(dimethylcarbamoyl)benzo[b]thiophen-5-yl)carbamoyl)-1-methyl-1H-pyrrol-3-yl)carbamate (30)

Obtained 0.130 g (reaction yield: 60%), as a brown solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ8.25 (d, J = 2.01 Hz, 1H), 7.70 - 7.81 (m, 2H), 7.46 (s, 1H), 7.36 (dd, J = 8.69 Hz, 2.14 Hz, 1H), 6.88 (br. s., 1H), 6.70 (br. s., 1H), 6.33 (br. s., 1H), 3.93 (s, 3H), 3.22 (br. s., 6H), 1.52 (s, 9H). ^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ164.8, 159.7, 153.5, 139.4, 138.7, 135.8, 135.2, 125.5, 123.3, 122.7, 122.0, 119.3, 118.8, 115.3, 103.9, 80.4, 36.8, 28.4. m/z (+ESI) calc. for $C_{22}H_{26}N_4O_4S$ $(M)^+$ 442.1 found 443.1 $([M]+H)^+$

Synthesis of benzofused side chains 31 to 38

The selected benzofused methyl ester intermediate **27** or **28** (1 equiv.) was dissolved in MeOH (8 mL), and an excess of NaOH 1M aqueous solution was added to the solution. The reaction mixture was left under magnetic stirrer overnight at room temperature until TLC showed total disappearance of the starting material. The solvent was evaporated under vacuum using a rotary evaporator, and citric acid 1M aqueous solution was added to the crude of reaction until acid pH was reached causing the simultaneous precipitation of a white solid. The precipitate was collected by filtration under vacuum and dissolved in DMF (4 mL). EDCI (2.5 equiv.) and DMAP (3 equiv.) were added to the solution and the reaction mixture was left under magnetic stirrer for 20 minutes at room temperature under N_2 atmosphere. Change in colour of the solution was observed from light yellow to dark brown. The correspondent amine (1.5 equiv.) was added to the solution and left under magnetic stirrer overnight. The reaction did not go to completion. The reaction was quenched by the addition of H_2O (10 mL) that was subsequently extracted with EtOAc (3 x 8 mL). The collected organic phases were washed sequentially with brine (10 mL), $NaHCO_3$ saturated aqueous solution (10 mL) and citric acid aqueous solution 0.1 M (10 mL). The organic phase was dried on MgSO4 and evaporated under vacuum using a rotary evaporator giving the correspondent crude of reaction that was purified by column chromatography on silica gel (mobile phase: from 100 DCM to 70/30, v/v, DCM/EA, depending on the substrate) to give the final products **33-38.**

In the case of diethyl derivatives **31** and **32** a different procedure was applied. After the hydrolysis of the methyl ester benzofused intermediates **27** and **28**, the obtained acid (1 equiv.) was dissolved in DMF (4 mL) and diethyl amine (2 equiv.) and DIPEA (2 equiv.) were added to the solution that was kept at 0^{O}C in an ice bath. At that point T_3P (50% solution in DMF, 2 equiv.) was added the reaction mixture that was allowed to reach room temperature and left under magnetic stirrer overnight. The reaction didn't go to completion and was quenched by addition of water (10 mL) and then extracted with ethyl acetate (3 x 8 mL). The organic phase was washed with brine (10 mL) and $NaHCO_3$ saturated aqueous solution (10 mL). The collected organic phase was dried on MgSO4

and subsequently evaporated under vacuum using a rotary evaporator. The crude of the reaction was subsequently purified by column chromatography (mobile phase: from 100 DCM to 70/30, v/v, DCM/EA) to give the final products **31** and **32**.

Tert-butyl (5-((2-(diethylcarbamoyl)benzofuran-5-yl)carbamoyl)-1-methyl-1H-pyrrol-3-yl)carbamate (31)

Obtained 0.036 g (reaction yield: 40%) as a white solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ7.95 (d, J = 2.01 Hz, 1H), 7.80 (s, 1H), 7.34 - 7.47 (m, 2H), 7.23 (s, 1H), 6.88 (br. s., 1H), 6.68 (br. s., 1H), 6.43 (br. s., 1H), 3.91 (s, 3H), 3.59 (br. s., 4H), 1.51 (s, 9H), 1.29 (br. s., 6H). ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ160.3, 159.8, 153.5, 151.5, 150.5, 133.8, 127.5, 123.3, 121.9, 119.9, 118.7, 113.6, 111.9, 111.3, 103.9, 80.3, 36.7, 28.4, 14.2. m/z (+ESI) calc. for $C_{24}H_{30}N_4O_5$ $(M)^+$ 454.2 found 455.1($[M]+H)^+$.

Tert-butyl (5-((2-(diethylcarbamoyl)benzo[b]thiophen-5-yl)carbamoyl)-1-methyl-1H-pyrrol-3-yl)carbamate (32)

Obtained 0.035 g (reaction yield: 41%) as a white solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ8.20 (d, *J* = 2.01 Hz, 1H), 7.92 (br. s., 1H), 7.69 (d, *J* = 8.81 Hz, 1H), 7.37 (s, 1H), 7.31 (d, *J* = 8.81 Hz, 1H), 6.90 (br. s., 1H), 6.70 (br. s., 1H), 6.55 (br. s., 1H), 3.89 (s, 3H), 3.55 (q, *J* = 7.22 Hz, 4H), 1.51 (s, 9H), 1.26 (t, *J* = 7.18 Hz, 6H). ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 164.2, 159.8, 153.5, 139.4, 138.8, 135.4, 135.3, 124.2, 123.2, 122.5, 122.0, 119.3, 118.9, 115.4, 80.2, 36.8, 28.4, 14.2. m/z (+ESI) calc. for $C_{24}H_{30}N_4O_4S$ $(M)^+$ 470.2 found 471.1 $([M]+H)^+$.

Tert-butyl (1-methyl-5-((2-(piperidine-1-carbonyl)benzofuran-5-yl)carbamoyl)-1*H*-pyrrol-3-yl)carbamate (33)

Obtained 0.031 g (reaction yield: 34%) as a white solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ7.91-7.93 (m, 2H), 7.42 (d, 1H), 7.34 - 7.39 (m, 1H), 7.13 (d, *J* = 0.76 Hz, 1H), 6.88 (s, 1H), 6.68 (s, 1H), 6.55 (s, 1H), 3.88 (s, 3H), 3.72 (br. s., 4H), 1.62 - 1.75 (m, 6H), 1.50 (s, 9H). ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ159.9, 159.8, 153.5, 151.3, 150.0, 133.9, 127.4, 123.3, 122.0, 120.0, 118.7, 113.5, 111.9, 111.2, 103.9, 80.2, 36.7, 28.4, 24.7, 24.6. m/z (+ESI) calc. for $C_{25}H_{30}N_4O_5$ $(M)^+$ 466.2 found 467.1 $([M]+H)^+$.

Tert-butyl (1-methyl-5-((2-(piperidine-1-carbonyl)benzo[b]thiophen-5-yl)carbamoyl)-1H-pyrrol-3-yl)carbamate (34)

Obtained 0.032 g (reaction yield: 33%) as a yellow solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ8.20 (s, 1H), 7.86 (s, 1H), 7.72 (d, *J* = 9.06 Hz, 1H), 7.30 - 7.37 (m, 2H), 6.90 (br. s., 1H), 6.70 (br. s., 1H), 6.48 (br. s., 1H), 3.91 (s, 3H), 3.68 (br. s., 4H), 1.59 - 1.75 (m, 6H), 1.52 (s, 9H). ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 163.7, 159.8, 153.5, 139.3, 138.2, 135.5, 135.3, 124.6, 123.3, 122.6, 122.0, 119.2, 118.8, 115.3, 104.0, 80.3, 36.8, 28.4, 24.6. m/z (+ESI) calc. for $C_{25}H_{30}N_4O_4S$ $(M)^+$ 482.2 found 483.0 $([M]+H)^+$.

Tert-butyl (1-methyl-5-((2-(morpholine-4-carbonyl)benzofuran-5-yl)carbamoyl)-1H-pyrrol-3-yl)carbamate (35)

Obtained 0.038 g (reaction yield: 40%) as a grey solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ8.01 (d, *J* = 2.01 Hz, 1H), 7.69 (s, 1H), 7.48 (d, *J* = 8.81 Hz, 1H), 7.41 (dd, *J* = 8.94 Hz, 2.14 Hz, 1H), 7.31 (d, *J* = 0.76 Hz, 1H), 6.87 (s, 1H), 6.69 (s, 1H), 6.29 (br. s., 1H), 3.93 (s, 3H), 3.89 (br. s.,4H), 3.80 (br. s., 4H), 1.52 (s, 9H). ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ159.6, 159.5, 151.4, 149.5, 133.9, 127.2, 123.2, 121.9, 120.0, 118.6, 116.3, 113.4, 112.6, 111.9, 103.7, 80.2, 66.9, 36.7, 28.3. m/z (+ESI) calc. for $C_{24}H_{28}N_4O_6$ $(M)^+$ 468.2 found 469.2 $([M]+H)^+$.

Tert-butyl (1-methyl-5-((2-(morpholine-4-carbonyl)benzo[b]thiophen-5-yl)carbamoyl)-1H-pyrrol-3-yl)carbamate (36)

Obtained 0.044 g (reaction yield: 48%) as a white solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ8.20 (d, *J* = 2.01 Hz, 1H), 7.92 (br. s., 1H), 7.69 (d, *J* = 8.81 Hz, 1H), 7.37 (s, 1H), 7.31 (d, *J* = 8.81 Hz, 1H), 6.90 (br. s., 1H), 6.70 (br. s., 1H), 6.55 (br. s., 1H), 3.89 (s, 3H), 3.55-370 (m, 8H), 1.51 (s, 9H). ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ163.9, 159.7, 153.5, 139.2, 137.2, 135.6, 135.4, 125.4, 123.3, 122.7, 122.0, 119.4, 118.8, 115.3, 104.0, 80.4, 66.9, 36.8, 28.4. m/z (+ESI) calc. for $C_{24}H_{28}N_4O_5S$ $(M)^+$ 484.1 found 485.1 ([M]+H)

Tert-butyl (1-methyl-5-((2-(thiomorpholine-4-carbonyl)benzofuran-5-yl)carbamoyl)-1H-pyrrol-3-yl)carbamate (37)

Obtained 0.039 g (reaction yield: 41%) as a white solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ8.01 (d, *J* = 2.01 Hz, 1H), 7.67 (s, 1H), 7.48 (d, *J* = 8.81 Hz, 1H), 7.40 (dd, *J* = 8.81 Hz, 2.27 Hz, 1H), 6.87 (s, 1H), 6.69 (s, 1H), 6.27 (br. s., 1H), 4.08 (br. s., 4H), 3.93 (s, 3H), 2.73 - 2.80 (m, 4H), 1.52 (s, 9H). ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ160.0, 159.7, 153.4, 151.5, 151.4, 149.6, 134.0, 127.4, 123.3, 120.1, 118.7, 113.5, 112.3, 112.1, 103.8, 80.4, 36.8, 28.4. m/z (+ESI) calc. for $C_{24}H_{28}N_4O_5S$ $(M)^+$ 484.1 found 485.1 $([M]+H)^+$.

Tert-butyl (1-methyl-5-((2-(thiomorpholine-4-carbonyl)benzo[b]thiophen-5-yl)carbamoyl)-1*H*-pyrrol-3-yl)carbamate (38)

Obtained 0.048 g (reaction yield: 50%) as a white solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ8.23 (s, 1H), 7.92 (s, 1H), 7.73 (d, *J* = 10.83 Hz, 1H), 7.33 - 7.40 (m, 2H), 6.87 (br. s., 1H), 6.71 (br. s., 1H), 6.45 (br. s., 1H), 4.00 (br. s., 4H), 3.91 (s, 3H), 2.71 (br. s., 4H), 1.51 (s, 9H). ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ164.2, 159.8, 153.5, 139.2, 137.3, 135.6, 125.0, 123.3, 122.6, 122.0, 119.5, 118.9, 116.5, 115.4, 104.1, 80.4, 36.8, 35.6, 28.4, 27.9. m/z (+ESI) calc. for $C_{24}H_{28}N_4O_4S_2$ $(M)^+$ 500.1 found 501.2 $([M]+H)^+$.

Synthesis of final compounds 3-12.

C8-boc protected tails **29-38** (1 equiv.) were dissolved in MeOH (3 mL), and HCl 4M in dioxane (3 mL) was added to the solution that was left under magnetic stirrer for 2 hours. TLC confirmed the deprotection of the amine. The reaction mixture was evaporated under vacuum using a rotary evaporator obtaining the formation of a light brown solid. PBD protected core **26** was dissolved in DMF (4 mL) and EDCI (2 equiv.) and DMAP (3 equiv.) were added to the solution that was left under magnetic stirrer in N_2 atmosphere for 20 minutes. The desired, deprotected, side chains was added to the reaction mixture that was left under magnetic stirrer overnight. TLC and LC-MS analysis showed the formation of the protected PBD-C8 derivative. H_2O (10 mL) was added to the reaction mixture to quench the reaction. The aqueous phase was subsequently extracted with EtOAc (3 x 10 mL). The collected organic phases were then washed with brine (10 mL), $NaHCO_3$ saturated aqueous solution (10 mL) and citric acid aqueous solution 0.1 M (10 mL). The collected organic phase was dried over MgSO4 and evaporated under vacuum using a rotary evaporator. The obtained crude of the reaction was purified by column chromatography (mobile phase: from DCM /acetone, 90/10, v/v to DCM/acetone, 60/40/, v/v depending on the substrate), to give protected PBD-conjugates. The obtained product (1 equiv.) was dissolved in DCM (4 mL) and Tetrakis Pd (0.05 equiv.), triphenylphosphine (0.25 equiv.) and pyrrolidine (1.2 equiv.) were added to the solution. The reaction mixture was kept under magnetic stirrer for 20 minutes until TLC showed completion of the reaction. The solvent was evaporated under vacuum using a rotary evaporator and subsequently under high vacuum to eliminate any residue of pyrrolidine. The crude of the reaction was purified by column chromatography (mobile phase: from DCM/acetone, 90/10, v/v to DCM/acetone, 40/60/, v/v, depending on the substrate) affording pure final compounds **3-12**.

(*S*)-N-(2-(dimethylcarbamoyl)benzofuran-5-yl)-4-(4-((7-methoxy-5-oxo-2,3,5,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)butanamido)-1-methyl-1H-pyrrole-2-carboxamide (3)

Obtained 0.045 g (reaction yield: 64%) as a white solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ8.28 (s, 1H), 8.27 (s, 1H), 7.99 (d, *J* = 1.76 Hz, 1H), 7.64 (d, *J* = 4.53 Hz, 1H), 7.48 (s, 1H), 7.36 - 7.46 (m, 2H), 7.20 (s, 1H), 7.15 (d, *J* = 1.76 Hz, 1H), 6.79 (s, 1H), 6.58 (d, *J* = 2.01 Hz, 1H), 3.97 - 4.08 (m, 2H), 3.87 (s, 3H), 3.84 (s, 3H), 3.76 (m, 1H), 3.66 - 3.72 (m, 1H), 3.51 - 3.57 (m, 1H), 3.32 (br. s., 3H), 3.13 (br. s., 3H), 2.24 - 2.33 (m, 2H), 2.17 - 2.22 (m, 2H), 1.93 - 2.08 (m, 4H). ^{13}C

NMR (100 MHz, CHLOROFORM-*d*) δ169.9, 164.7, 162.8, 160.9, 160.0, 151.4, 150.6, 149.7, 147.7, 140.62, 134.1, 127.2, 123.0, 121.7, 120.4, 120.3, 119.9, 113.7, 111.8, 111.6, 110.8, 104.1, 68.1, 56.1, 53.8, 46.7, 36.8, 32.9, 29.5, 29.3, 24.9, 24.2. HRMS (ESI, m/z): calc. for $C_{34}H_{36}N_6O_7$ $([M]+H)^+$ 641.2718 found 641.2717.

(*S*)-N-(2-(diethylcarbamoyl)benzofuran-5-yl)-4-(4-((7-methoxy-5-oxo-2,3,5,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)butanamido)-1-methyl-1H-pyrrole-2-carboxamide (4)

Obtained 0.025 g (reaction yield: 56%) as a white solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ 8.20 (s, 1H), 8.16 (s, 1H), 7.98 (d, *J* = 1.51 Hz, 1H), 7.65 (d, *J* = 4.28 Hz, 1H), 7.50 (s, 1H), 7.41 - 7.45 (m, 2H), 7.22 (s, 1H), 7.15 (s, 1H), 6.80 (s, 1H), 6.57 (s, 1H), 4.07 (t, *J* = 5.67 Hz, 2H), 3.88 (s, 3H), 3.86 (s, 3H), 3.73 - 3.82 (m, 2H), 3.67 - 3.73 (m, 1H), 3.60 (br. s., 4H), 2.45 - 2.53 (m, 2H), 2.25 - 2.34 (m, 2H), 2.18 - 2.24 (m, 2H), 1.99 - 2.07 (m, 2H), 1.28 (br. s., 6H). ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ169.9, 164.6, 160.3, 160.0, 151.4, 150.7, 150.3, 147.7, 140.6, 134.1, 127.4, 123.0, 121.6, 120.4, 120.2, 119.9, 113.7, 111.8, 111.7, 111.2, 110.9, 104.1, 69.5, 68.1, 56.1, 53.8, 46.7, 36.8, 31.8, 29.6, 29.3, 24.9, 24.2, 16.2. HRMS (ESI, m/z): calc. for $C_{36}H_{40}N_6O_7$ $([M]+H)^+$ 669.3031 found 669.3029.

(*S*)-4-(4-((7-methoxy-5-oxo-2,3,5,11a-tetrahydro-1H-benzo[*e*]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)butanamido)-1-methyl-N-(2-(piperidine-1-carbonyl)benzofuran-5-yl)-1H-pyrrole-2-carboxamide (5)

Obtained 0.020 g (reaction yield: 46%) as a white solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ8.18 (s, 1H), 8.13 (s, 1H), 7.98 (s, 1H), 7.65 (d, *J* = 4.28 Hz, 1H), 7.50 (s, 1H), 7.42 - 7.45 (m, 2H), 7.13 - 7.16 (m, 2H), 6.80 (s, 1H), 6.56 (d, *J* = 2.01 Hz, 1H), 4.06 (t, *J* = 6.17 Hz, 2H), 3.88 (s, 3H), 3.86 (s, 3H), 3.67 - 3.78 (m, 6H), 3.52 -3.59 (m, 1H), 2.46 - 2.52 (m, 2H), 2.27 - 2.32 (m, 2H), 2.19 - 2.24 (m, 2H), 2.00 - 2.07 (m, 2H), 1.62 - 1.71 (m, 6H). ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ169.9, 164.6, 162.8, 160.0, 151.3, 150.7, 149.5, 147.7, 140.6, 134.1, 127.4, 123.0, 121.6, 120.4, 120.1, 119.8, 113.6, 111.8, 111.7, 111.1, 110.9, 104.0, 69.5, 56.1, 53.8, 46.7, 31.8, 29.3, 24.9, 24.6, 24.2. HRMS (ESI, m/z): calc. for $C_{37}H_{41}N_6O_7$ $([M]+H)^+$ 681.3031 found 681.3029.

(*S*)-4-(4-((7-methoxy-5-oxo-2,3,5,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)butanamido)-1-methyl-N-(2-(morpholine-4-carbonyl)benzofuran-5-yl)-1H-pyrrole-2-carboxamide (6)

Obtained 0.030 g (reaction yield: 58%) as a white solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ 8.04 (d, *J* = 1.51 Hz, 1H), 8.00 (s, 1H), 7.70 (s, 1H), 7.67 (d, *J* = 4.53 Hz, 1H), 7.54 (s, 1H), 7.46 - 7.50 (m, 2H), 7.30 (s, 1H), 7.12 (d, *J* = 1.76 Hz, 1H), 6.84 (s, 1H), 6.59 (d, *J* = 2.01 Hz, 1H), 4.15 (t, *J* = 5.92 Hz, 2H), 3.92 (s, 3H), 3.91 (s, 3H), 3.76 - 3.83 (m, 8H), 3.70 - 3.75 (m, 2H), 3.53 - 3.60 (m, 1H), 2.56 (t, *J* = 6.80 Hz, 2H), 2.23 - 2.34 (m, 4H), 2.00 - 2.10 (m, 2H). ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ169.9, 164.6, 162.8, 159.9, 151.5, 149.4, 140.7, 134.2, 127.4, 127.3, 123.1, 121.5, 120.4, 119.9, 119.5, 119.4, 118.8, 115.8, 113.9, 113.6, 112.7, 112.0, 111.8, 111.2, 104.1, 68.1, 67.0, 56.2, 53.8, 46.7, 36.8, 29.6, 25.0, 24.2. HRMS (ESI, m/z): calc. for $C_{36}H_{39}N_6O_8$ $([M]+H)^+$ 683.2824 found 683.2827.

(*S*)-4-(4-((7-methoxy-5-oxo-2,3,5,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)butanamido)-1-methyl-N-(2-(thiomorpholine-4-carbonyl)benzofuran-5-yl)-1H-pyrrole-2-carboxamide (7)

Obtained 0.030 g (reaction yield: 48%) as a light yellow solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ8.28 (s, 1H), 8.13 (s, 1H), 8.00 (d, *J* = 2.01 Hz, 1H), 7.64 (d, *J* = 4.28 Hz, 1H), 7.45 - 7.49 (m, 2H), 7.39 - 7.43 (m, 1H), 7.21 (s, 1H), 7.11 (d, *J* = 1.76 Hz, 1H), 6.79 (s, 1H), 6.60 (d, *J* = 1.76 Hz, 1H), 3.99 - 4.09 (m, 6H), 3.87 (s, 3H), 3.84 (s, 3H), 3.74 - 3.81 (m, 1H), 3.66 - 3.71 (m, 1H), 3.49 - 3.57 (m, 1H), 2.70 - 2.77 (m, 4H), 2.46 - 2.52 (m, 2H), 2.25 - 2.32 (m, 2H), 1.93 - 2.08 (m, 4H). ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ169.9, 164.6, 162.8, 160.0, 159.9, 151.3, 150.6, 149.4, 147.8, 140.6, 134.3, 127.2, 123.0, 121.6, 120.5, 120.4, 119.8, 113.6, 112.3, 111.8, 104.2, 68.1, 56.1, 53.8, 46.7, 36.8, 32.9, 29.5, 29.3, 24.9, 24.2. HRMS (ESI, m/z): calc. for $C_{36}H_{39}N_6O_7S$ $([M]+H)^+$ 699.2595 found 699.2597.

(*S*)-N-(2-(dimethylcarbamoyl)benzo[b]thiophen-5-yl)-4-(4-((7-methoxy-5-oxo-2,3,5,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)butanamido)-1-methyl-1H-pyrrole-2-carboxamide (8)

Obtained 0.040 g (reaction yield: 67%) as a light yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.98 (s, 1H), 9.93 (s, 1H), 8.43 (d, *J* = 2.01 Hz, 1H), 7.91 (d, *J* = 8.81 Hz, 1H), 7.76 - 7.80 (m, 2H),

7.68 (dd, *J* = 8.81 Hz, 2.01 Hz, 1H), 7.33 (s, 1H), 7.24 (d, *J* = 1.76 Hz, 1H), 7.01 (d, *J* = 1.76 Hz, 1H), 6.83 (s, 1H), 4.09 - 4.16 (m, 1H), 4.00 - 4.07 (m, 1H), 3.85 (s, 3H), 3.83 (s, 3H), 3.65-3.68 (m, 1H), 3.57 - 3.63 (m, 1H), 3.33 - 3.42 (m, 1H), 3.25 (br. s., 3H), 3.06 (br. s., 3H), 2.41 - 2.47 (m, 2H), 2.18 - 2.29 (m, 2H), 2.02 - 2.08 (m, 2H), 1.88 - 1.98 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.9, 164.2, 163.3, 163.2, 159.8, 150.1, 146.9, 140.5, 139.2, 138.5, 136.6, 133.9, 126.0, 122.5, 122.2, 122.1, 119.9, 119.8, 118.9, 115.3, 111.2, 104.8, 68.5, 55.8, 53.4, 46.3, 36.2, 32.1, 29.6, 28.8, 24.4. HRMS (ESI, m/z): calc. for $C_{34}H_{37}N_6O_6S$ $([M]+H)^+$ 657.2490 found 657.2487.

(*S*)-N-(2-(diethylcarbamoyl)benzo[b]thiophen-5-yl)-4-(4-((7-methoxy-5-oxo-2,3,5,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)butanamido)-1-methyl-1H-pyrrole-2-carboxamide (9)

Obtained 0.031 g (reaction yield: 62%) as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.94 (s, 1H), 9.98 (s, 1H), 8.45 (s, 1H), 7.91 (d, *J* = 8.78 Hz, 1H), 7.75 (d, *J* = 4.40 Hz, 1H), 7.57 - 7.70 (m, 2H), 7.33 (s, 1H), 7.24 (s, 1H), 7.02 (s, 1H), 6.83 (s, 1H), 4.09 - 4.20 (m, 1H), 3.96 - 4.09 (m, 1H), 3.85 (s, 3H), 3.83 (s, 3H), 3.63 - 3.71 (m, 2H), 3.55 - 3.63 (m, 1H), 3.49 (br. s., 4H), 2.40 - 2.46 (m, 2H), 2.16 - 2.26 (m, 2H), 1.99 - 2.05 (m, 2H), 1.90-1.95 (m, 2H), 1.19 (br. s., 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ168.6, 162.8, 159.8, 158.3, 154.6, 151.7, 150.0, 147.9, 143.3, 140.8, 139.2, 138.5, 136.6, 134.9, 133.7, 129.9, 127.0, 124.4, 123.4, 122.5, 122.2, 122.0, 119.8, 118.9, 116.4, 115.4, 110.6, 68.5, 55.8, 36.3, 30.7, 29.6, 24.4, 16.0. HRMS (ESI, m/z): calc. for $C_{36}H_{41}N_6O_6S$ $([M]+H)^+$ 685.2803 found 685.2808.

(*S*)-4-(4-((7-methoxy-5-oxo-2,3,5,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)butanamido)-1-methyl-N-(2-(piperidine-1-carbonyl)benzo[b]thiophen-5-yl)-1H-pyrrole-2-carboxamide (10)

Obtained 0.030 g (reaction yield: 69%) as a yellow solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ 8.26 (d, *J* = 1.76 Hz, 1H), 8.10 (s, 1H), 7.99 (s, 1H), 7.75 (d, *J* = 8.56 Hz, 1H), 7.66 (d, *J* = 4.53 Hz, 1H), 7.52 (s, 1H), 7.42 (dd, *J* = 8.69 Hz, 2.14 Hz, 1H), 7.38 (s, 1H), 7.15 (d, *J* = 1.51 Hz, 1H), 6.82 (s, 1H), 6.56 (d, *J* = 1.76 Hz, 1H), 4.09 (t, *J* = 6.17 Hz, 2H), 3.90 (s, 3H), 3.89 (s, 3H), 3.77 - 3.84 (m, 2H), 3.68 (br. s., 5H), 2.48 - 2.55 (m, 2H), 2.27 - 2.34 (m, 2H), 2.20 - 2.27 (m, 2H), 2.00 - 2.09 (m, 2H), 1.55 - 1.69 (m, 6H). HRMS (ESI, m/z): calc. for $C_{37}H_{41}N_6O_6S$ $([M]+H)^+$ 697.2803 found 697.2806.

(*S*)-4-(4-((7-methoxy-5-oxo-2,3,5,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)butanamido)-1-methyl-N-(2-(morpholine-4-carbonyl)benzo[b]thiophen-5-yl)-1H-pyrrole-2-carboxamide (11)

Obtained 0.030 g (reaction yield: 48%) as a yellow solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ 8.29 (s, 1H), 8.10 (s, 1H), 7.82 (s, 1H), 7.77 (d, *J* = 8.56 Hz, 1H), 7.67 (d, *J* = 4.28 Hz, 1H), 7.53 (s, 1H), 7.47 (d, *J* = 9.06 Hz, 1H), 7.42 (s, 1H), 7.13 (s, 1H), 6.83 (s, 1H), 6.60 (s, 1H), 4.12 (t, *J* = 5.79 Hz, 2H), 3.91 (s, 3H), 3.90 (br. s., 4H), 3.79 (br. s., 9H), 3.54 - 3.62 (m, 1H), 2.50 - 2.58 (m, 2H), 2.28-2.32 (m, 2H), 2.21 - 2.27 (m, 2H), 2.00 - 2.10 (m, 2H). ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ 170.0, 164.6, 163.9, 162.8, 153.6, 150.6, 147.7, 140.7, 137.1, 132.7, 130.7, 125.4, 123.0, 122.7, 121.5, 120.6, 120.0, 119.7, 115.5, 111.8, 111.1, 110.5, 68.1, 66.9, 56.2, 53.5, 46.7, 36.8, 33.1, 25.0, 24.2. HRMS (ESI, m/z): calc. for $C_{36}H_{39}N_6O_7S$ $([M]+H)^+$ 699.2995 found 699.2596.

(*S*)-4-(4-((7-methoxy-5-oxo-2,3,5,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)butanamido)-1-methyl-N-(2-(thiomorpholine-4-carbonyl)benzo[b]thiophen-5-yl)-1H-pyrrole-2-carboxamide (12)

Obtained 0.024 g (reaction yield: 57%) as a yellow solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ8.29 (s, 1H), 8.11 (s, 1H), 7.83 (s, 1H), 7.76 (d, *J* = 8.56 Hz, 1H), 7.67 (d, *J* = 4.28 Hz, 1H), 7.53 (s, 1H), 7.44 - 7.49 (m, 1H), 7.40 (s, 1H), 7.13 (s, 1H), 6.84 (s, 1H), 6.60 (s, 1H), 4.08 - 4.15 (m, 2H), 4.01 (br. s., 4H), 3.91 (s, 3H), 3.90 (s, 3H), 3.73-3.82 (m, 1H), 3.68 - 3.74 (m, 1H), 3.52 - 3.61 (m, 1H), 2.72 (br. s., 4H), 2.49 - 2.57 (m, 2H), 2.27 - 2.35 (m, 2H), 2.19 - 2.26 (m, 2H), 1.98 - 2.09 (m, 2H). ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ170.0, 164.6, 164.1, 162.7, 150.6, 147.7, 140.6, 139.2, 137.3, 135.7, 135.4, 125.0, 123.1, 122.6, 121.5, 120.6, 120.0, 119.7, 116.2, 115.5, 111.8, 111.1, 104.2, 68.1, 56.2, 53.8, 46.7, 36.8, 33.1, 29.6, 29.3, 25.0, 24.2. HRMS (ESI, m/z): calc. for $C_{36}H_{39}N_6O_6S_2$ $([M]+H)^+$ 715.2367 found 715.2367.

Synthesis of methyl 4-(4-((tert-butoxycarbonyl)amino)phenyl)-1-methyl-1H-pyrrole-2-carboxylate (39)

Commercially available methyl 4-bromo-1-methyl-1H-pyrrole-2-carboxylate (1.0 g, 4 mmol, 1 equiv.) was dissolved in a mixture of ACN (40 mL) and H_2O (36 mL), and commercially available (4-((tert-butoxycarbonyl)amino)phenyl)boronic acid (1.1 equiv.) and K_2CO_3 (3 equiv.) were added to the solution that was transferred to a microwave vial. The reaction mixture was degassed using N_2. Tetrakis Pd (0.05 equiv.) was added to the reaction mixture that was then heated under microwave radiation at 100° C for 6 minutes. TLC and LC-MS analysis showed the formation of the product, but the reaction did not go to completion. The reaction mixture was filtered under vacuum through a path of Celite and washed with AcOEt. The filtrate was evaporated under vacuum using a rotary evaporator. The obtained solid residue was purified by column chromatography on silica gel (mobile phase: DCM/AcOEt, 80/20, v/v) giving pure **39** (0.635 g, reaction yield: 42%) as an amber oil. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 7.47 - 7.52 (m, 1H), 7.41 - 7.44 (m, 2H), 7.32 - 7.38 (m, 2H), 7.17 (d, *J* = 2.01 Hz, 1H), 7.04 (d, *J* = 2.27 Hz, 1H), 3.96 (s, 3H), 3.85 (s, 3H), 1.53 (s, 9H). ^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ: 161.9, 153.1, 136.8, 135.7, 129.4, 127.2, 126.2, 123.5, 119.2, 114.7, 80.5, 51.1, 37.2, 28.4. m/z (+ESI) calc. for $C_{18}H_{22}N_2O_4$ $(M)^+$ 330.1 found 331.0 $([M]+H)^+$.

Synthesis of methyl 4-(4-(4-((tert-butoxycarbonyl)amino)-1-methyl-1H-pyrrole-2-carboxamido)phenyl)-1-methyl-1H-pyrrole-2-carboxylate (40)

Compound **39** (1.2 equiv.) was dissolved in MeOH (7 mL), and HCl 4M in Dioxane (7 mL) was added to the solution that was left under magnetic stirrer for 2 hours until TLC showed completion of the reaction. The reaction mixture was then evaporated under vacuum using a rotary evaporator.

Commercially available 4-((tert-butoxycarbonyl)amino)-1-methyl-1H-pyrrole-2-carboxylic acid (0.659 g, 2.7 mmol, 1 equiv.) was dissolved in DMF (7 mL) and EDCI (2.4 equiv.) and DMAP (3 equiv.) were added to the solution that was left to stir in N_2 atmosphere for 30 minutes. The deprotected compound **39** was added to the reaction mixture and left to stir overnight at room temperature in N_2 atmosphere. The reaction did not go to completion and was quenched by the addition of H_2O (10 mL). The aqueous phase was then extracted with AcOEt (3 x 10 mL). The organic phase was sequentially washed with Citric Acid 0.1 M aqueous solution (10 mL), saturated $NaHCO_3$ aqueous solution (10 mL) and Brine (10 mL). The collected organic phase was dried over $MgSO_4$ and evaporated under vacuum using a rotary evaporator. The crude of the reaction was

purified by column chromatography on silica gel (mobile phase: DCM/AcOEt, 60/40, v/v) giving pure compound **40** (0.621 g, reaction yield: 50%) as a white solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 7.64 (s, 1H), 7.53 (d, *J* = 8.81 Hz, 2H), 7.45 (d, *J* = 8.81 Hz, 2H), 7.19 (d, *J* = 2.01 Hz, 1H), 7.06 (d, *J* = 2.27 Hz, 1H), 6.85 (br. s., 1H), 6.64 (br. s., 1H), 6.31 (br. s., 1H), 3.96 (s, 3H), 3.91 (s, 3H), 3.85 (s, 3H), 1.52 (s, 9H). ^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ: 161.7, 159.7, 153.4, 140.5, 136.3, 134.4, 130.4, 126.2, 123.6, 123.0, 122.0, 118.9, 114.8, 112.4, 103.8, 80.3, 50.9, 37.0, 36.7, 28.2. m/z (+ESI) calc. for $C_{24}H_{28}N_4O_5$ $(M)^+$ 452.2 found 453.1 $([M]+H)^+$.

Synthesis of MPB side chains 41 to 43

Starting from intermediate **40**, the same procedure used for the synthesis and purification of compound **33-38** was applied to give compounds **41-43**.

Tert-butyl (1-methyl-5-((4-(1-methyl-5-(piperidine-1-carbonyl)-1H-pyrrol-3-yl)phenyl)carbamoyl)-1H-pyrrol-3-yl)carbamate (41)

Obtained 0.060 g (reaction yield: 50%) as light yellow solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 7.67 (s, 1H), 7.45 - 7.54 (m, 2H), 7.40 (d, *J* = 8.56 Hz, 2H), 6.93 (d, *J* = 1.76 Hz, 1H), 6.87 (br. s., 1H), 6.62 (br. s., 1H), 6.54 (d, *J* = 1.76 Hz, 1H), 6.47 (br. s., 1H), 3.90 (s, 3H), 3.77 (s, 3H), 3.65 - 3.73 (m, 4H), 1.71 (br. s., 2H), 1.60 - 1.66 (m, 4H), 1.51 (s, 9H). ^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ: 162.7, 159.5, 153.4, 135.8, 130.9, 126.6, 125.4, 123.5, 122.8, 122.28, 121.9, 120.3, 118.5, 109.2, 103.7, 80.2, 47.9, 36.7, 35.7, 28.3, 25.7, 24.7. m/z (+ESI) calc. for $C_{28}H_{35}N_5O_4$ $(M)^+$ 505.2 found 506.2 $([M]+H)^+$.

Tert-butyl (1-methyl-5-((4-(1-methyl-5-(morpholine-4-carbonyl)-1H-pyrrol-3-yl)phenyl)carbamoyl)-1H-pyrrol-3-yl)carbamate (42)

Obtained 0.087 g, (reaction yield: 72%) as a yellow-orange solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 7.70 (br. s., 1H), 7.50 (d, *J* = 7.05 Hz, 2H), 7.36 (d, *J* = 7.55 Hz, 2H), 6.97 (br. s., 1H), 6.85 (br. s., 1H), 6.65 (br. s., 1H), 6.55 (br. s., 1H), 6.46 (br. s., 1H), 3.90 (br. s., 3H), 3.80 (br. s., 7H), 3.74 (br. s., 4H), 1.51 (br. s., 9H). ^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ: 163.1, 159.5, 153.8, 140.9, 136.2, 130.7, 125.4, 123.5, 122.9, 122.1, 122.0, 120.3, 118.5, 110.2, 103.8, 80.3, 67.1, 38.6, 36.5, 35.7, 28.4. m/z (+ESI) calc. for $C_{27}H_{33}N_5O_5$ $(M)^+$ 507.2 found 508.1 $([M]+H)^+$.

Tert-butyl (1-methyl-5-((4-(1-methyl-5-(thiomorpholine-4-carbonyl)-1H-pyrrol-3-yl)phenyl)carbamoyl)-1H-pyrrol-3-yl)carbamate (43)

Obtained 0.040 g (reaction yield: 86%) as a yellow oil. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 7.64 (br. s., 1H), 7.52 (d, *J* = 8.06 Hz, 2H), 7.36 (d, *J* = 8.06 Hz, 2H), 6.97 (s, 1H), 6.85 (br. s., 1H), 6.64 (br. s., 1H), 6.54 (s, 1H), 6.38 (br. s., 1H), 4.04 (br. s., 4H), 3.91 (s, 3H), 3.78 (s, 3H), 2.71 (br. s., 4H), 1.51 (br. s., 9H). ^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ: 163.1, 159.5, 153.4, 135.9, 130.6, 125.8, 125.5, 123.5, 123.0, 122.9, 121.8, 120.3, 118.6, 109.6, 103.7, 80.4, 36.7, 35.8, 28.3, 27.9. m/z (+ESI) calc. for $C_{27}H_{33}N_5O_4S$ $(M)^+$ 523.2 found 524.1 $([M]+H)^+$.

Synthesis of Bromo-pyrrole intermediates 44 and 45

Starting from commercially available N-methyl Bromo-pyrrole methyl esterthe same procedure used for the synthesis and purification of compound **21** (after basic hydrolysis of the methyl ester) was applied to give intermediates **44-45.**

The crude of reaction was purified by column chromatography on silica gel (mobile phase: DCM/AcOEt, 80/20, v/v), giving pure compounds **44** and **45**.

4-bromo-N,N,1-trimethyl-1H-pyrrole-2-carboxamide (44)

Obtained 0.420 g (reaction yield: 74%) as a light yellow solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 6.67 (s, 1H), 6.34 (s, 1H), 3.74 (s, 3H), 3.12 (br. s., 6H). ^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ: 163.1, 126.2, 125.2, 114.9, 94.0, 35.8. m/z (+ESI) calc. for $C_8H_{11}BrN_2O$ $(M)^+$ 230.0 found 230.9 $([M]+H)^+$.

4-bromo-N,N-diethyl-1-methyl-1H-pyrrole-2-carboxamide (45)

Obtained 0.484 g (reaction yield: 77%) as a light brown solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 6.65 (d, *J* = 1.76 Hz, 1H), 6.28 (d, *J* = 1.76 Hz, 1H), 3.68 (s, 3H), 3.49 (q, *J* = 7.22 Hz, 4H), 1.19 (t, *J* = 7.18 Hz, 6H). ^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ: 162.7,

126.7, 124.9, 112.6, 94.2, 42.5, 35.9, 11.2. m/z (+ESI) calc. for $C_{10}H_{15}BrN_2O$ $(M)^+$ 258.0 found 260.9 $([M]+H)^+$.

Synthesis of MPB derivatives 46 and 47

Starting from intermediate **44-45**, the same procedure used for the synthesis and purification of compound **39** was applied to give intermediates **46-47**.

Tert-butyl (4-(5-(dimethylcarbamoyl)-1-methyl-1H-pyrrol-3-yl)phenyl) carbamate (46)

Obtained 0.360 g (reaction yield: 61%) as an orange-brown solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 7.36 - 7.41 (m, 2H), 7.30 - 7.36 (m, 2H), 6.93 (d, *J* = 2.01 Hz, 1H), 6.60 (d, *J* = 2.01 Hz, 1H), 6.52 (s, 1H), 3.81 (s, 3H), 3.17 (s, 6H), 1.53 (s, 9H). ^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ: 164.1, 152.8, 136.2, 130.0, 126.2, 125.2, 122.8, 122.7, 119.1, 110.4, 80.5, 36.2, 28.4. m/z (+ESI) calc. for $C_{19}H_{25}N_3O_3$ $(M)^+$ 343.1 found 344.0 $([M]+H)^+$.

Tert-butyl (4-(5-(diethylcarbamoyl)-1-methyl-1H-pyrrol-3-yl)phenyl)carbamate (47)

Obtained 0.313 g (46%) ad a light yellow solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 7.72 - 7.76 (m, 2H), 7.66 - 7.72 (m, 2H), 7.27 (s, 1H), 7.03 (s, 1H), 6.91 (d, *J* = 2.01 Hz, 1H), 4.12 (s, 3H), 3.93 (q, *J* = 7.05 Hz, 4H), 1.89 (s, 9H), 1.61 (t, *J* = 7.18 Hz, 6H). ^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ: 163.7, 152.7, 135.8, 129.9, 127.0, 125.7, 123.1, 121.6, 118.7, 108.4, 80.2, 35.5, 28.4, 13.7. m/z (+ESI) calc. for $C_{21}H_{29}N_3O_3$ $(M)^+$ 371.22 found 372.1 $([M]+H)^+$.

Synthesis of MPB tails 48-49

Starting from intermediates **46-47**, the same procedure used for the synthesis and purification of compound **40** was applied for to give intermediates **48-49**

Tert-butyl (5-((4-(5-(dimethylcarbamoyl)-1-methyl-1H-pyrrol-3-yl)phenyl)carbamoyl)-1-methyl-1H-pyrrol-3-yl)carbamate (48)

Obtained 0.185 g (reaction yield: 95%) as a brown solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 7.65 (s, 1H), 7.50 (d, *J* = 8.56 Hz, 2H), 7.38 (d, *J* = 8.56 Hz, 2H), 6.94 (d, *J* = 1.76 Hz, 1H), 6.86 (s, 1H), 6.62 (s, 2H), 6.43 (br. s., 1H), 3.90 (s, 3H), 3.80 (s, 3H), 3.18 (br. s., 6H), 1.51 (s, 9H). ^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ: 163.9, 159.7, 153.6, 136.0, 130.8, 126.4, 125.5, 124.1, 123.5, 122.6, 121.9, 120.6, 118.6, 110.4, 103.5, 80.4, 37.0, 35.8, 28.2. m/z (+ESI) calc. for $C_{25}H_{31}N_5O_4$ $(M)^+$ 465.2 found 466.1 $([M]+H)^+$.

Tert-butyl (5-((4-(5-(diethylcarbamoyl)-1-methyl-1H-pyrrol-3-yl)phenyl)carbamoyl)-1-methyl-1H-pyrrol-3-yl)carbamate (49)

Obtained 0.090 g (reaction yield: 53%) as a light yellow solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 7.64 (s, 1H), 7.50 (d, *J* = 8.56 Hz, 2H), 7.39 (d, *J* = 8.56 Hz, 2H), 6.93 (d, *J* = 2.01 Hz, 1H), 6.86 (s, 1H), 6.61 (s, 1H), 6.57 (d, *J* = 2.01 Hz, 1H), 6.47 (br. s., 1H), 3.90 (s, 3H), 3.76 (s, 3H), 3.58 (q, *J* = 6.88 Hz, 4H), 1.51 (s, 9H), 1.22 - 1.27 (m, 6H). ^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ: 163.3, 159.8, 153.8, 136.0, 131.4, 127.1, 124.9, 124.0, 122.7, 122.0, 121.9, 120.2, 118.7, 108.0, 103.8, 80.3, 37.3, 35.4, 28.3, 14.0. m/z (+ESI) calc. for $C_{27}H_{35}N_5O_4$ $(M)^+$ 493.2 found 494.1 $([M]+H)^+$.

Synthesis of final compounds 13-17

Starting from the appropriate intermediates, the same procedure used for the synthesis and purification of compound **3-12** was applied to give final compounds **13-17**.

(*S*)-4-(4-(4-(4-((7-methoxy-5-oxo-2,3,5,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)butanamido)-1-methyl-1H-pyrrole-2-carboxamido)phenyl)-N,N,1-trimethyl-1H-pyrrole-2-carboxamide (13)

Obtained 0.044 g (reaction yield: 70%) as a light yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.90 (s, 1H), 9.76 (s, 1H), 7.77 (d, *J* = 4.53 Hz, 1H), 7.62 - 7.69 (d, *J* = 8.81 Hz, 2H), 7.44 - 7.50 (d, *J* = 8.56 Hz, 2H), 7.33 (s, 1H), 7.30 (d, *J* = 1.76 Hz, 1H), 7.20 (d, *J* = 1.76 Hz, 1H), 6.95 (d, *J* = 1.76 Hz, 1H), 6.82 (s, 1H), 6.75 (d, *J* = 1.76 Hz, 1H), 4.08 - 4.17 (m, 1H), 3.99 - 4.08 (m, 1H), 3.82 (s, 6H), 3.68 (s, 4H), 3.55 - 3.63 (m, 1H), 3.37-3.41 (m, 1H), 3.07 (br. s., 6H), 2.44 (t, *J* = 7.43 Hz, 2H), 2.15 - 2.33 (m, 2H), 2.00 - 2.07 (m, 2H), 1.92 (d, *J* = 5.54 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 168.6, 164.4, 163.3, 162.4, 159.3, 150.4, 146.7, 140.7, 139.0, 136.9, 129.6, 126.2, 124.1, 122.7, 122.6, 121.8, 120.5, 115.8, 114.4, 119.8, 111.2, 110.1, 109.2, 104.5, 67.3, 54.8, 53.7, 46.2, 36.0, 31.8, 28.5, 24.7, 23.4. HRMS (ESI, m/z): calc. for $C_{37}H_{41}N_7O_6$ $([M]+H)^+$ 680.3191 found 680.3196.

(*S*)-N,N-diethyl-4-(4-(4-(4-((7-methoxy-5-oxo-2,3,5,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)butanamido)-1-methyl-1H-pyrrole-2-carboxamido)phenyl)-1-methyl-1H-pyrrole-2-carboxamide (14)

Obtained 0.029 g (reaction yield: 64%) as a white solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 8.22 (br. s., 1H), 7.86 (br. s., 1H), 7.66 (d, *J* = 4.53 Hz, 1H), 7.51 (s, 1H), 7.44 - 7.49 (d, *J* = 8.56 Hz, 2H), 7.33 - 7.41 (d, *J* = 8.81 Hz, 2H), 7.17 (d, *J* = 1.76 Hz, 1H), 6.94 (d, *J* = 1.51 Hz, 1H), 6.82 (s, 1H), 6.59 (d, *J* = 2.01 Hz, 1H), 6.35 (d, *J* = 1.51 Hz, 1H), 4.10 (t, *J* = 6.17 Hz, 2H), 3.89 (s, 6H), 3.76-3.80 (m, 1H), 3.74 (s, 3H), 3.68 - 3.72 (m, 1H), 3.53 - 3.62 (m, 5H), 2.49-2.53 (m, 2H), 2.26 - 2.33 (m, 2H), 2.23 (t, *J* = 6.67 Hz, 2H), 1.98 - 2.09 (m, 2H), 1.25 (t, *J* = 7.05 Hz, 6H).^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ: 170.0, 165.0, 163.7, 162.8, 160.1, 150.7, 147.9, 140.6, 136.2, 130.7, 127.4, 125.5, 122.9, 121.5, 119.6, 112.1, 110.8, 108.4, 104.0, 68.2, 56.4, 53.7, 47.0, 36.9, 35.8, 33.1, 29.9, 25.2, 24.3, 18.0. HRMS (ESI, m/z): calc. for $C_{39}H_{45}N_7O_6$ $([M]+H)^+$ 708.3504 found 708.3508.

(*S*)-4-(4-((7-methoxy-5-oxo-2,3,5,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)butanamido)-1-methyl-N-(4-(1-methyl-5-(piperidine-1-carbonyl)-1H-pyrrol-3-yl)phenyl)-1H-pyrrole-2-carboxamide (15)

Obtained 0.021 g (reaction yield: 81%) as a light brown solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 8.06 (s, 1H), 7.84 (s, 1H), 7.66 (d, *J* = 4.53 Hz, 1H), 7.52 (s, 1H), 7.46 - 7.51 (d, *J* = 8.31 Hz, 2H), 7.34 - 7.43 (d, *J* = 8.56 Hz, 2H), 7.13 - 7.19 (m, 1H), 6.95 (d, *J* = 1.76 Hz, 1H), 6.83 (s, 1H), 6.56 (d, *J* = 1.51 Hz, 1H), 6.40 (d, *J* = 1.51 Hz, 1H), 4.11 (t, *J* = 5.92 Hz, 2H), 3.90 (s, 6H), 3.78 - 3.84 (m, 1H), 3.76 (s, 3H), 3.69 - 3.73 (m, 4H), 3.54-3.63 (m, 2H), 2.50 - 2.57 (m, 2H), 2.27 - 2.35 (m, 2H), 2.20 - 2.26 (m, 2H), 1.98 - 2.09 (m, 2H), 1.66-1.75 (m, 6H). ^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ: 169.9, 164.6, 162.8, 160.3, 159.7, 150.7, 147.8, 140.7, 136.1, 130.7, 130.5, 126.8, 125.4, 123.3 123.0, 122.4, 121.6, 120.7, 120.5, 119.8, 111.9, 111.1, 109.1, 103.5, 68.1, 56.4, 53.7, 46.6, 36.9, 35.6, 32.8, 29.7, 25.0, 24.7, 24.3. HRMS (ESI, m/z): calc. for $C_{40}H_{45}N_7O_6$ $([M]+H)^+$ 720.3504 found 720.3505.

(*S*)-4-(4-((7-methoxy-5-oxo-2,3,5,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)butanamido)-1-methyl-N-(4-(1-methyl-5-(morpholine-4-carbonyl)-1H-pyrrol-3-yl)phenyl)-1H-pyrrole-2-carboxamide (16)

Obtained 0.032 g (reaction yield: 50%) as a light yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.91 (s, 1H), 9.78 (s, 1H), 7.78 (br. s., 1H), 7.61 - 7.71 (m, 2H), 7.44 - 7.52 (m, *J* = 7.81 Hz, 2H), 7.34 (br. s., 2H), 7.21 (s, 1H), 6.96 (s, 1H), 6.83 (s, 1H), 6.71 (s, 1H), 4.10 - 4.16 (m, 1H), 4.00 - 4.08 (m, 1H), 3.83 (s, 6H), 3.70 (s, 3H), 3.64 (br. s., 10H), 3.56 - 3.60 (m, 1H), 2.44 (t, *J* = 7.05 Hz, 2H), 2.15 - 2.25 (m, 2H), 1.99 - 2.15 (m, 2H), 1.88 - 1.98 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 168.9, 164.3, 163.4, 162.0, 159.5, 150.3, 146.9, 140.7, 137.0, 129.6, 125.5, 124.5, 123.2, 123.0, 122.7, 122.0, 121.9, 120.3, 11.3, 110.o, 109.0, 104.6, 67.8, 66.3, 55.4, 54.8, 46.5, 36.4, 31.8, 29.7, 28.7, 24.8, 23.7. HRMS (ESI, m/z): calc. for $C_{39}H_{43}N_7O_7$ $([M]+H)^+$ 722.3297 found 722.3302.

(*S*)-4-(4-((7-methoxy-5-oxo-2,3,5,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)butanamido)-1-methyl-N-(4-(1-methyl-5-(thiomorpholine-4-carbonyl)-1H-pyrrol-3-yl)phenyl)-1H-pyrrole-2-carboxamide (17)

Obtained 0.077 g (reaction yield: 96%) as a light brown solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 7.98 (s, 1H), 7.92 (s, 1H), 7.66 (d, *J* = 4.53 Hz, 1H), 7.53 (d, *J* = 8.56 Hz, 2H), 7.51 (s, 1H), 7.38 (d, *J* = 8.56 Hz, 2H), 7.14 (d, *J* = 1.76 Hz, 1H), 6.97 (d, *J* = 1.76 Hz, 1H), 6.82 (s, 1H), 6.55 (d, *J* = 1.76 Hz, 1H), 6.45 (d, *J* = 2.01 Hz, 1H), 4.09 (t, *J* = 6.42 Hz, 2H), 4.03 (dd, *J* = 7.05 Hz, 2.77 Hz, 4H), 3.89 (s, 3H), 3.88 (s, 3H), 3.78 - 3.83 (m, 1H), 3.77 (s, 3H), 3.68 - 3.74 (m, 1H), 3.51 - 3.61 (m, 1H), 2.67 - 2.73 (m, 4H), 2.48 - 2.54 (m, 2H), 2.27 - 2.35 (m, 2H), 2.20 - 2.26 (m, 2H), 2.00 - 2.08 (m, 2H). ^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ: 169.8, 164.6, 163.1, 162.7, 159.6, 150.6, 147.7, 140.6, 136.1, 130.4, 125.8, 125.4, 123.3, 123.0, 122.9, 121.5, 120.5, 120.4, 119.8, 111.6, 110.9, 109.5, 103.7, 68.1, 64.5, 56.1, 53.7, 46.7, 36.7, 35.8, 32.9, 29.5, 29.2, 27.9, 24.9, 24.2. HRMS (ESI, m/z): calc. for $C_{39}H_{43}N_7O_7$ $([M]+H)^+$ 738.3068 found 738.3075.

Bacterial strains

Bacterial strains used in this study are listed in Tables 1 and 2. All isolates were cultured in tryptic soy broth (TSB) and on TSA plates at 37°C. All chemicals were sourced from Sigma-Aldrich unless otherwise stated. Strains from the *P. aeruginosa* PA01 [25] and *K. pneumoniae* MKP103 transposon [26] mutant libraries were obtained from the University of Washington, Genome services, WA, USA.

Susceptibility testing

The minimal inhibitory concentration (MIC) was determined using the microdilution broth method[27]. Compounds were initially dissolved in DMSO prior to dilution in broth. Equivalent concentrations of solvent had no effect on bacterial growth. The MIC was defined as the lowest concentration of compound which resulted in no visible growth at an optical density of 600 nm. Experiments were performed in triplicate.

Time-kill curve analysis

Three independent repeats were performed for each strain with each compound. 10 mL of fresh TSB media was inoculated with ~1x10^7 CFU/mL of the test organism. Bacteria were challenged at 4 x MIC for each compound and incubated at 37 °C in a rotary shaker at 200 rpm. 100 µL aliquots

were taken at 0, 1, 2, 4, 6 and 24 hours post inoculation and serial dilutions performed in sterile PBS. Total viable counts were determined by Miles-Misra dilutions method. The compound is considered bactericidal if the inoculum was reduced >3 log10 CFU/mL and bacteriostatic if inoculum was decreased by 0-3 log10 CFU/mL.

Efflux and influx assays

An adapted microdilution broth method was performed to evaluate the efflux potential of the compounds. In this assay, 50 µL/well of efflux pump inhibitor (EPI) at 4 x final concentration and 50 µL/well of test organism at 2 x 10^5 CFU/mL were added to wells containing 100 µL/well of a dilution series of compound. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was added at a final concentration of 10 mg/L and phenylalanine-arginine beta-naphthylamide PAβN at 25 mg/L. TSB media was supplemented with $MgSO_4$ (40 µM) to prevent the permeabilisation of the outer membrane of Gram-negative bacteria by PAβN [18]. A decrease in MIC of the compound of at least four-fold was defined as significant for efflux activity [28].

The influx assay was performed exactly as described for the efflux assay, with the addition of Polymyxin B Nonapeptide (PMBN) at a final concentration of 30 mg/L.

Transposon mutants in efflux pump genes in *P. aeruginosa* strain PA01 were generated as described previously [29] and provided via http://www.gs.washington.edu/labs/manoil/libraryindex.html.

Gyrase inhibition assay

The effect of the antimicrobial agents on gyrase activity was assessed using the *S. aureus* and *E. coli* gyrase supercoiling gel based assays obtained from Inspiralis (Norwich, UK) [30]. Methods were conducted as per the manufacturer's instructions.

Whole genome sequence analysis and transposon mutants

NCTC 13368 isolates resistant to compound **7** were analysed to identify the site of mutations. Isolates were passaged 10 times in the absence of selection and checked to confirm the resistance phenotype was maintained. DNA was isolated and the genome sequenced by PHE-GSDU on an Illumina (HiSeq 2500) as previously described [31]. Potential individual mutations were identified using Galaxy [32].

Cell culture and MTT assay

WI-38 cell line was obtained from the American Type Culture Collection. The cells were grown in normal conditions in an incubator at 37 °C in a humidified atmosphere containing 5% CO_2. The appropriate medium (EMEM or MEM, Gibco) supplemented with fetal bovine serum (10%, v/v, Sigma Aldrich) was used for culturing the cells. The viability assay was conducted in a 96-well plate, and the plates were left under continuous incubation with the drug for 24 hours. The media was removed, MTT reagent added and finally the absorbance of the formazan crystals was read using a plate reader (Envision Plate Reader, PerkinElmer). The values of absorbance obtained were normalized with the blank and then used for the determination of the % of viability compared to the control. For single point toxicity screening, the number of repeats (n) was equal to 6, and the result is reported as average values.

FRET-based DNA melting assay

The modified, fluorophore-tagged hairpin oligonucleotides were purchased from Eurogentec. The assay was carried out using a previously published procedure[7] with 5:1 ligand oligonucleotide ratio. The working solution of 400 nM was made in FRET buffer (50 mM potassium cacodylate, pH 7.4) and annealed by heating the working solution at 90 °C for 6 minutes followed by cooling to room temperature and storage at this temperature for 5 hours before the assay. The compound and oligonucleotide were combined in a 1:1 ratio (25 µL of each) in a 96-well plate and was heated in the range of 30-100 °C after 3 hours incubation at 25 °C. The fluorescence readings (excitation 490 nm, emission 520 nm) were taken at fixed intervals of 0.5 °C, over the cited range. The data obtained by the instrument were processed through normalisation of the curve using Origin 7.0 (OriginLab Corp., USA). The DNA melting temperature values were then determined using a pre-set script that determined the values as the point of inflection of the first derivative of the curve. Each experiment was conducted in triplicate and the values reported are the ΔTm average along with the standard deviation of the mean.

Mutation frequency

Mutation frequencies were determined following the method of Evans and Titlow (1998). Briefly, 100 µL of bacterial culture, grown to an OD_{600} of approximately 0.5 - 0.6 was used to inoculate the surface of TSB agar containing compounds at a range of concentrations above and below the MIC. The plates were then incubated at 37 ^{0}C and the plates assessed for the presence of colonies following 48 hour incubation. Miles-Misra was also performed on drug free TSA plates to determine the exact number of CFU/mL in the initial culture. Mutation frequencies were then

calculated by dividing the number of colonies on plates containing the specific agents by the total number of CFU that were plated.

Reporter strain assays

Strains from the *E. coli* K12 MG1655 promoter library created by [20] were purchased from Dharmacon (GE Life Sciences). Strains were maintained on 25 mg/L kanamycin TSA plates and cultured in M9 media supplemented with 1% glucose, 0.2% Casamino acids, 0.5 mg/L thiamine, 100 μM $CaCl_2$, 2 mM $MgSO_4$ and 25 mg/L kanamycin [21]. Two-fold serial dilutions of compounds were made up in DMSO to 50 X the required concentration, with a previously determined inhibitory concentration in the middle of the series. 2 μL of the dilution series was added per well, with 2 μL of DMSO in 'untreated' wells, then 98 μL of overnight culture back diluted to an OD600 of 0.2 was added to each well. Strain U66 or U139, containing the promoterless plasmids pUA66 or pUA139 respectively, was included for every compound tested as a negative control. Plates were incubated at 37°C with 200 rpm in a CLARIOstar microplate reader (BMG Labtech). Fluorescence (RFU) and cell growth (OD600) were measured every 30 minutes for 20 hours. Fluorescence data was normalised by cell density (RFU/OD600) at 9 hours, where fluorescence became stable, and at the highest concentration of compound for which there was growth. Fold induction of the promoter was calculated by dividing the fluorescence of the treated sample by fluorescence of the untreated sample.

Molecular docking of the compounds to gyrase A:

AutoDock SMINA was used for molecular docking of compounds **7** and **8** to the minimized crystal structure of Gyrase A from *S. aureus* (PDB ID 2XCT), and cryo EM structure of Gyrase A from *E. coli* (PDB ID 6RKS) for finding the best binding pocket by exploring all probable binding cavities in the enzyme. All the parameters were kept in their default values. Then, GOLD molecular docking was used for molecular docking of the compounds into the SMINA-located binding site for performing flexible molecular docking and determining more precise and evaluated energies and scores[33,34]. Based on the fitness function score and ligand binding position, the best-docked pose for each compound was selected.

ASSOCIATED CONTENT

Supporting information.

Supporting information are available containing:

-Docking poses of selected compounds with DNA gyrase, LC-Ms method, NMR spectra and HRMS (PDF)

-Molecular formula strings (CSV)

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Notes

The authors declare no competing financial interest.

The PDB ID code for DNA Gyrase in complex with 7 and 8 is 2XCT. Authors will release the atomic coordinates and experimental data upon article publication.

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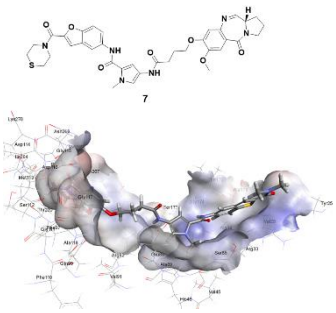
ABBREVIATIONS USED

ESBL, Extended Spectrum Beta Lactamase; MDR, multi-drug resistant; PDR, Pan-drug resistant; HTS, high-throughput screening; MRSA, methicillin resistant *S. aureus;* VRE, vancomycin resistant *Enterococci;* HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIPEA, N,N-diisopropylethylamine; FRET, fluorescence resonance energy transfer; FAM, 6-Carboxyfluorescein; RND, resistance nodulation division; TAMRA, 5-Carboxytetramethylrhodamine.

Graphical abstract



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